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Initiation of Proliferative Events by Human α-Thrombin Requires Both Receptor Binding and Enzymic Activity

Darrell H. Carney, Janet Stiernberg, and John W. Fenton II

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550 (D.H.C., J.S.) and Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201 (J.W.F.)

To determine the role of thrombin high-affinity receptor occupancy and enzymic activity in thrombin initiation of cell proliferation, we have utilized thrombin derivatives which separate these functions. We previously showed that enzymically active γ-thrombin stimulates ion fluxes without binding to high-affinity sites, whereas proteolytically inhibited DIP-α-thrombin which binds to high-affinity receptors does not. Since neither derivative initiates DNA synthesis by itself, this suggested that two separate sequences of events might be necessary for a complete initiation signal. We now report that the combination of DIP-α-thrombin and γ-thrombin initiate DNA synthesis and cell proliferation to levels approaching the maximal initiation by native α-thrombin. This combinatory effect is dose-dependent for both γ-thrombin and DIP-α-thrombin in the same concentration range as α-thrombin alone. Thus, these same concentrations of α-thrombin alone may be required to initiate each sequence of events. The combinatory stimulation could be achieved even if the derivatives were added individually up to 8 hr apart. Moreover, preincubation with either derivative shortened the lag period for initiation of DNA synthesis by native α-thrombin. These results indicate that both receptor occupancy and enzymic activity are necessary for thrombin initiation of cell proliferation and that each action initiates a sequence of early events which moves the cell forward toward entry into a proliferative cycle.

Key words: thrombin, growth factors, receptor occupancy, growth control, cell cycle, wound healing

Highly purified human thrombin initiates cell proliferation without the addition of serum or other purified growth factors [1–4]. Fibroblastlike cells have high-affinity thrombin receptors [5,6] which appear to be clustered on the surface of the cells prior to thrombin binding [7–9]. These receptor clusters do not associate with coated pits and do not appear to participate in rapid receptor-mediated internalization and degradation [7,8]. Some internalization and degradation of thrombin does occur in these cells, but it is mediated by protease-nexin (PN), a protein that complexes with thrombin or other serine proteases in the medium and then binds to a separate PN

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receptor for rapid degradation [10,11]. Thus, the thrombin receptors may be quite different from other receptors involved in ligand internalization and degradation.

Previous studies have shown that thrombin action at the cell surface is sufficient to initiate cell proliferation [12,13] and that the internalization and degradation of thrombin through PN-mediated binding is not involved in initiation [14]. Thus, it would appear that thrombin initiates cell proliferation by some form of transmembrane signal. Generation of this signal appears to require thrombin interaction with its high-affinity receptors [5] and continued receptor occupancy for up to 8 hr [15]. The requirement for continued receptor occupancy has also been observed with other growth factors [16,17] and is consistent with data showing that a number of late events including amiloride-sensitive ion fluxes [18] and microtubule rearrangements [19] must occur 6–8 hr after growth factor addition to allow initiation of a proliferative cycle.

Several studies have suggested that enzymic activity of thrombin is necessary to initiate cell proliferation. Proteolytically inactivated thrombin derivatives that retain their ability to bind to high-affinity thrombin receptors are not able to initiate cell proliferation [6,20]. In addition, there appears to be a correlation between thrombin initiation of chick embryo cell proliferation and loss of an iodinated cell surface protein with M_r = 43,000 [21]. These studies suggested that thrombin cleavage of the receptor or an adjacent protein is involved in initiation of cell proliferation. More recent studies have suggested that enzymic activity may be even more important than receptor occupancy since saturation of high-affinity thrombin binding sites with proteolytically inactivated diisopropylphospho (DIP)-conjugated α-thrombin does not inhibit initiation by native α-thrombin [22].

We recently used various thrombin derivatives to evaluate the relationship between thrombin enzymic activity, receptor binding, and stimulation of ion fluxes [23]. In these studies, we found that DIP-α-thrombin binding to the thrombin receptor was not sufficient to stimulate ion fluxes, but that proteolytically active thrombin derivatives that could not bind to the high-affinity sites stimulated both early and late ion fluxes [23]. None of these derivatives, however, initiated cell proliferation by themselves. This suggested to us that two separate cell surface interactions might be involved in initiation. To pursue this possibility, we attempted to initiate DNA synthesis and cell proliferation in mouse embryo (ME) cells with combinations of these derivatives. We now report that by combining DIP-α-thrombin, which binds high-affinity sites, with γ-thrombin, which retains its esterase activity, ME cell proliferation and DNA synthesis are initiated to levels comparable to that achieved with native α-thrombin. These studies show that initiation by thrombin requires both receptor binding and enzymic activity and that these actions can be separated spatially and temporally.

MATERIALS AND METHODS

Materials

Dulbecco-Vogt modified Eagle's (DV) medium, Ham's F-12 medium, trypsin solution, glutamine, and antibiotics were purchased from Grand Island Biological Company (Grand Island, NY). Calf serum was purchased from Irvine Scientific (Santa Ana, CA). [^{3}H]thymidine (60 Ci/mmole) was purchased from ICN Pharmaceuticals (Irvine, CA); Na[^{125}I] (IMS-30) was obtained from Amersham (Arlington

Heights, IL); and human anti-thrombin III was purchased from Sigma (St. Louis, MO). All other common chemicals were purchased from Sigma or Fisher unless otherwise indicated.

Thrombin Preparations

Human α-thrombin was prepared from crude human serum fraction III paste (obtained through the courtesy of Dr. Bryan H. Landis, Armour Pharmaceutical Company, Kankakee, IL) and characterized as previously described [24,25]. The α-thrombin preparation used in these studies was a homogeneous preparation (100% α-thrombin) with high enzyme activity (Table I). γ-Thrombin was prepared by autocatalytic digestion of α-thrombin (2 mg/ml) during a 3-day incubation at 23°C in 3 M NaCl, pH 8 [26,27]. The resulting γ-thrombin preparation was dialyzed against 0.75 M NaCl and shown to retain less than 1% of its initial clotting activity. Thrombin inactivated at its active site serine was made by treatment with diisopropylphosphofluoridate (DIP-F) (Aldrich Chemical Company, Milwaukee, WI). DIP-F was added in molar excess to the enzyme in 0.75 M NaCl, 10 mM Tris, pH 8.3, at 23°C and repeatedly added at 10- to 15-min intervals until the fibrinogen clotting activity was less than 0.1% of the native α-thrombin preparation. The unconjugated hydrolyzed DIP-F product was removed by dialyzing against several changes of 0.75 M NaCl at 4°C. An M_r of 36,500 for α-thrombin was assumed for all thrombin forms [24,25].

Thrombin Activity Measurements

Specific fibrinogen clotting activities, expressed in U.S. (NIH) units per milligram of protein, were determined by measuring fibrin clot formation in a purified fibrinogen solution as previously described [24,28]. Active site titrations with the esterase substrate *p*-nitrophenyl-*p*-guanidinoenoate hydrochloride (NPGP) were performed as described [24]. Activities of the preparations used are given in Table I.

Iodination of Thrombin

Thrombin was iodinated using lactoperoxidase as described previously [5,7]. Using a 1:1 molar ratio of radioactive to nonradioactive iodine, thrombin was prepared with specific activities up to 4.3×10^4 CPM/ng, corresponding to 1.1 iodines per thrombin. The proteolytic activity of each iodinated thrombin preparation was determined by its ability to convert fibrinogen to fibrin as described above. All iodinated thrombin preparations retained >80% of their initial clotting activity.

TABLE I. Modifications and Enzymic Activities of Thrombin Preparations*

Preparation	Modification	Specific clotting activity (units/mg)	Active-site titration with NPGP (% active enzyme)
α-Thrombin	None	3,736	100.0
γ-Thrombin	Fibrin exosite B-chain fragmented into thirds	0.84	83.4
DIP-α-Thrombin	Catalytic site DIP-group conjugated to the catalytic serine	0.38	Negligible (< 1.0)

*See text for details.

Cell Culture

Primary cultures of mouse embryo (ME) cells were prepared weekly from the body walls of 12- to 17-day-old NIH-Swiss outbred mouse embryos as described previously [2] and cultured in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium (DV/F12) supplemented with 10% calf serum, penicillin (100 mg/ml), and streptomycin (100 μg/ml).

^{125}I-α-Thrombin Binding

^{125}I-α-thrombin binding to ME cells was performed on monolayer cultures of nonproliferating ME cells, as described elsewhere [5]. Cultures were rinsed and allowed to equilibrate with binding medium (serum-free DV medium containing 0.5% bovine serum albumin (BSA) buffered with 15 mM *N*-2-hydroxyethylpiperazine-*N*'2-ethane sulfonic acid (HEPES) at pH 7.0) for 20 min at 23°C. The medium was then changed to binding medium containing 20 ng/ml ^{125}I-α-thrombin. Incubation was continued for 90 min at 23°C. The binding assays were terminated by quickly rinsing the cells four times with cold PBS. The cells were then dissolved in 1.0 ml of 0.5 N NaOH for measurement of total radioactivity. It should be noted that under these conditions there is little, if any, protease-nexin (PN)-thrombin complex binding to the ME cells.

Initiation of DNA Synthesis and Cell Proliferation

To determine the ability of thrombin or various thrombin derivatives to initiate proliferative events, indicated concentrations of α-, γ-, or DIP-α-thrombin were added to quiescent serum-free cultures of ME cells. Initiation of DNA synthesis was determined by measuring the incorporation of [^{3}H]thymidine into cold trichloroacetic acid (TCA)-precipitable material during a 30-min incubation period generally from 23.5 to 24 hr after thrombin addition. Following incubation, cell monolayers were extracted (10 min) in 10% TCA and rinsed five times with cold 10% TCA. The acid-precipitable material was dissolved overnight in 0.5 ml of 0.5 N KOH at 23°C then 0.26 ml of 1 N HCl was added, and the solution was counted in 10 ml of Ready Solv-MP scintillation fluid (Beckman Instruments, Houston, TX).

To determine the ability of thrombin or various thrombin derivatives to initiate cell proliferation, serum-free ME cell cultures were incubated with thrombin or thrombin derivative preparations for 48 hr. The cells were then removed by trypsinization and counted in a Coulter electronic particle counter.

RESULTS

Properties of Thrombin Derivatives

Enzymatic activities of thrombin and the thrombin derivatives in these studies are shown in Table I. Native α-thrombin had a high specific fibrinogen clotting activity (3,736 NIH units/mg) and was 100% active as judged by active site titration. γ-Thrombin was prepared by autolytic cleavage of the α-thrombin B-chain as described in Methods. This enzyme retains esterase activity similar to α-thrombin (83.4%) but is exosite-inhibited, which prevents its binding and cleavage of fibrinogen. Thus, γ-thrombin has virtually no fibrinogen clotting activity (0.84 NIH units/mg). DIP-α-thrombin, prepared by conjugation of DIP to serine 205 of the

α-thrombin B-chain, is active site inhibited and thus has both very low fibrinogen-clotting activity (0.38 NIH units/mg) and low esterase activity (less than 1%).

Competition of Thrombin Derivatives for High-Affinity Binding Sites

As shown in Figure 1, α-thrombin has specific high-affinity binding sites on these ME cells. In competition binding studies using 20 ng/ml ^{125}I-α-thrombin, unlabeled native α-thrombin competes for approximately 70% of cell-associated ^{125}I-thrombin, and unlabeled DIP-α-thrombin, which is active-site-inhibited, competes as well as α-thrombin for binding of ^{125}I-α-thrombin. Thus, consistent with previous results [6,20], these results demonstrate that DIP-α-thrombin binds to the high-affinity α-thrombin binding sites with an affinity equivalent to that of α-thrombin. In contrast, γ-thrombin at concentrations up to 2 μg/ml did not compete for ^{125}I-α-thrombin binding. Thus, γ-thrombin does not appear to exhibit any high-affinity binding to these sites.

DIP- and γ-Thrombin Together Initiate DNA Synthesis and Cell Proliferation

Previous studies have shown that neither γ-thrombin nor DIP-α-thrombin initiates DNA synthesis or cell proliferation by itself [6,20,23], but that γ-thrombin, which does not bind to the high-affinity sites, can stimulate both early and late ion fluxes [23]. To determine whether the two different interactions (binding and stimulation of ion fluxes) together could initiate DNA synthesis, we looked at the combined effect of adding γ-thrombin and DIP-α-thrombin to quiescent ME cell cultures.

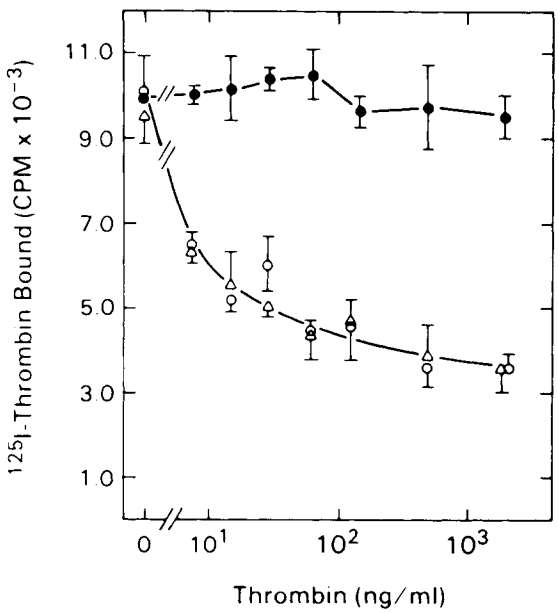

Fig. 1. ^{125}I-Thrombin binding: competition of unlabeled α-thrombin or thrombin derivatives. ^{125}I-thrombin (20 ng/ml) was incubated with quiescent monolayers of ME cells in the presence of the indicated concentrations of unlabeled α-thrombin (○), DIP-α-thrombin (△), or γ-thrombin (●). After 90 min, plates were rinsed and total cell-associated radioactivity determined (see Methods). Error bars represent one standard deviation from the mean of triplicate determinations.

As shown in Figure 2A, addition of γ-thrombin alone to quiescent serum-free cultures of ME cells caused only a slight increase in thymidine incorporation at concentrations up to 2 μg per ml. Similarly 2 μg per ml of DIP-α-thrombin (which would saturate the high-affinity α-thrombin binding sites) by itself did not stimulate thymidine incorporation. In contrast, when 2 μg per ml of DIP-α-thrombin was added to cultures with increasing concentrations of γ-thrombin, thymidine incorporation was stimulated to levels approaching the maximal stimulation of thymidine incorporation by α-thrombin. For example, at a γ-thrombin concentration of 2 μg/ml the stimulation observed in combination with DIP-α-thrombin was 85% of the maximal stimulation by α-thrombin (2 μg/ml) in the same experiment.

Based on comparison of the γ- and DIP-α-thrombin fibrinolytic activity, the DIP-α-thrombin preparation used for these experiments retained a maximum of 0.01% or in the present experiment, 0.2 ng/ml of α-thrombin activity (see Table I). To determine if residual α-thrombin could account for the observed initiation, we added a subinitiating concentration of native α-thrombin (15 ng/ml) to ME cells together with increasing concentrations of γ-thrombin. As shown in Figure 2B, this addition, which represents a 75-fold excess over the estimated residual thrombin, had little if any effect on initiation by γ-thrombin at concentrations up to 2 μg/ml. Thus, the synergistic stimulation between γ- and DIP-α-thrombin does not appear to be due to residual levels of native α-thrombin in these derivative preparations.

To further establish that the combinatory stimulation could not be caused by residual α-thrombin or active α-thrombin that might be liberated during incubation, we repeated these experiments in the presence of purified anti-thrombin III. As shown

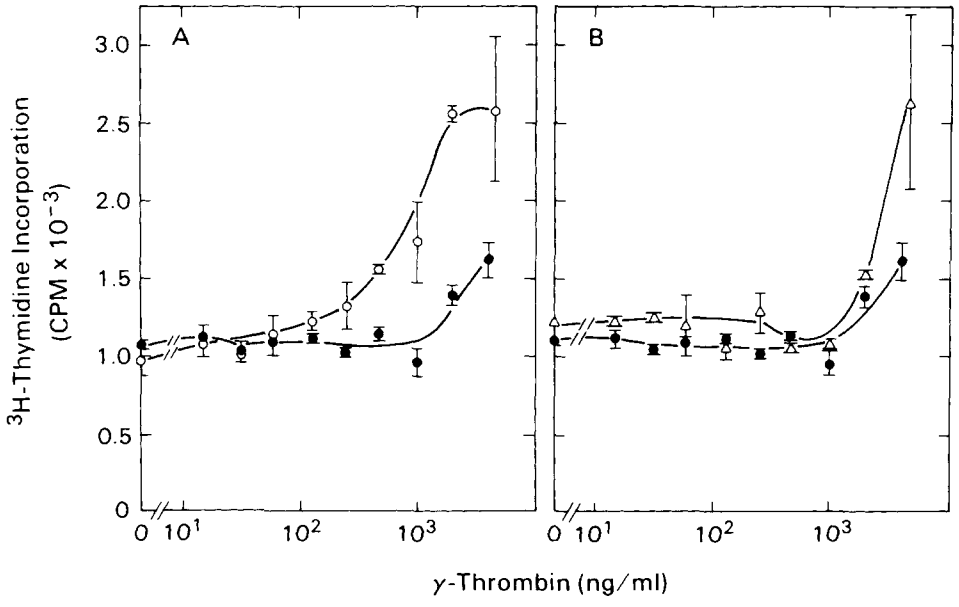

Fig. 2. Stimulation of thymidine incorporation by combinations of γ-thrombin and DIP-α-thrombin. Indicated concentrations of γ-thrombin were added to quiescent serum-free cultures of ME cells alone (●) or in the presence of 2 μg/ml of DIP-α-thrombin (panel A, ○) or 15 ng/ml of native α-thrombin (panel B, △). [^{3}H]Thymidine incorporation was determined 24 hr later as described in Methods.

in Figure 3A, anti-thrombin III concentrations of 0.1 and 0.4 units per milliliter inhibit the stimulation of thymidine incorporation by native α-thrombin with an almost complete inhibition of stimulation by up to 250 ng/ml α-thrombin at 0.4 units of anti-thrombin III. These concentrations of anti-thrombin III also inhibit the slight stimulation by addition of γ- or DIP-α-thrombin alone (Fig. 3B). In contrast, the combinatory stimulation observed by addition of γ- and DIP-α-thrombin to the same cultures, which was comparable to that achieved with α-thrombin, was only partially inhibited by 0.4 units per milliliter of anti-thrombin III. These effects are directly compared in Figure 3B. As shown, 125 ng/ml of α-thrombin stimulates thymidine incorporation to a level comparable to that achieved by combination of γ- and DIP-α-thrombin. This stimulation is almost completely inhibited, however, by 0.1 and 0.4 units per milliliter of anti-thrombin III, whereas the stimulation by γ- and DIP-α-thrombin is only partially inhibited at these concentrations. Thus, the stimulation of thymidine incorporation by γ- and DIP-α-thrombin cannot be explained either by low levels of residual α-thrombin in these preparations or by active thrombin liberation during incubation.

To assure ourselves that the combination of derivatives was actually initiating cell proliferation and not simply altering thymidine transport, experiments similar to that described above were performed to look for increases in cell number after 48 hr. As shown in Figure 4, 0.5 μg/ml of α-thrombin caused an 85% increase in cell number during this period. γ-Thrombin alone caused little if any increase at 0.5 μg/ml, and a 40% increase at 2 μg/ml. In combination with 2 μg/ml of DIP-α-thrombin,

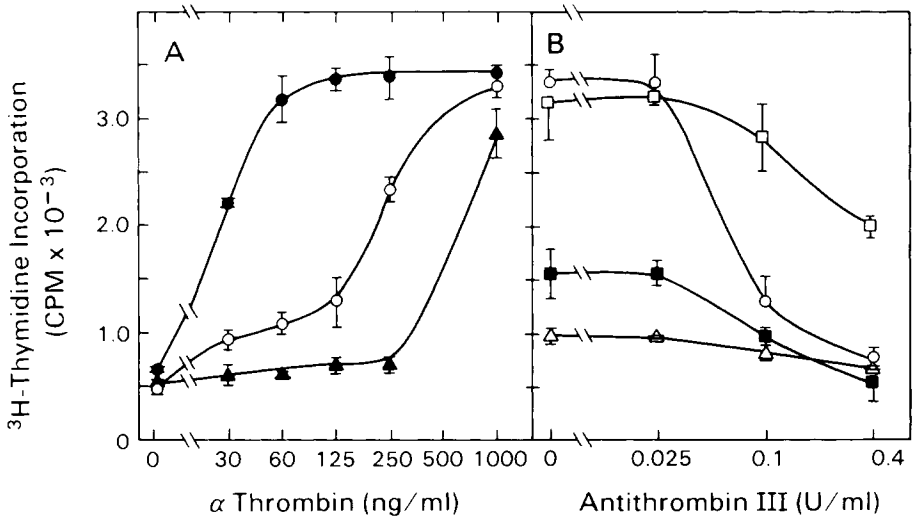

Fig. 3. Effect of anti-thrombin III on stimulation of thymidine incorporation by α-thrombin and by the combination of γ- and DIP-α-thrombin. Indicated concentrations of human anti-thrombin III and various thrombin forms were added to quiescent serum-free cultures of ME cells and [^{3}H]thymidine incorporation was determined 24 hr later as described in Methods. A) Native α-thrombin alone (●) or native α-thrombin in the presence of 0.1 units/ml (○) or 0.4 units/ml (▲) of purified human anti-thrombin III. B) 2 μg/ml DIP-α-thrombin alone (△); 1 μg/ml γ-thrombin alone (■); 125 ng/ml α-thrombin alone (○) or the combination of 2 μg/ml DIP-α-thrombin and 1 μg/ml γ-thrombin (□). Anti-thrombin III units are equivalent to the amount of anti-thrombin III in 1.0 ml of pooled human serum.

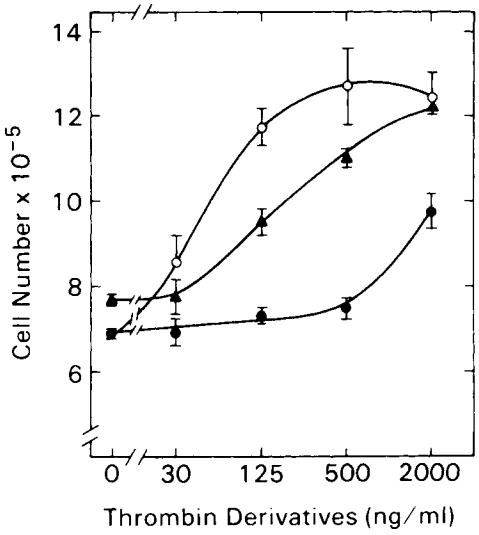

Fig. 4. Stimulation of cell proliferation by α-thrombin and thrombin derivatives. Indicated concentrations of native α-thrombin (○) or γ-thrombin with (▲) or without (●) DIP-α-thrombin (2 μg/ml) were added to quiescent serum-free cultures of ME cells and incubated for 48 hr. Cell number was determined as described in Methods.

γ-thrombin caused a 60% increase at 1 μg/ml and an increase equivalent to that of α-thrombin if both were added at 2 μg/ml. Thus, the synergistic stimulation of thymidine incorporation by DIP-α- and γ-thrombin appears to represent true initiation of DNA synthesis and cell proliferation.

To determine the DIP-α-thrombin concentration dependence of this effect, various concentrations of DIP-thrombin were added to quiescent cultures of ME cells in the presence or absence of 0.5 μg/ml of γ-thrombin (Fig. 5). As shown, DIP-α-thrombin alone did not initiate DNA synthesis, and in combination with γ-thrombin, DIP-α-thrombin has little effect at concentrations of 60 and 125 ng/ml. At 250 ng/ml, a concentration where more than 50% of the receptors would be occupied [5,20], there was significant stimulation with increasing stimulation at concentrations up to 2 μg/ml where the thrombin receptors would be saturated [5,20]. Thus, the synergistic stimulation is concentration-dependent for DIP-α-thrombin, and appears to require concentrations of DIP-α-thrombin that at least half-saturate the high-affinity α-thrombin binding sites.

Temporal Relationship Between γ- and DIP-α-Thrombin-Initiated Events

The above studies demonstrated that addition of γ- and DIP-α-thrombin derivatives initiated events leading to a stimulation of DNA synthesis and cell proliferation which nearly equaled that seen with native α-thrombin. Thus, initiation by thrombin appears to require two separate thrombin actions—receptor occupancy and enzymic activity. Since these actions can be physically separated on two different thrombin forms, each activity may initiate a separate sequence of events required for initiation. To pursue this idea, we attempted to separate these events temporally to determine if one sequence necessarily preceded the other or if they had to occur concomitantly.

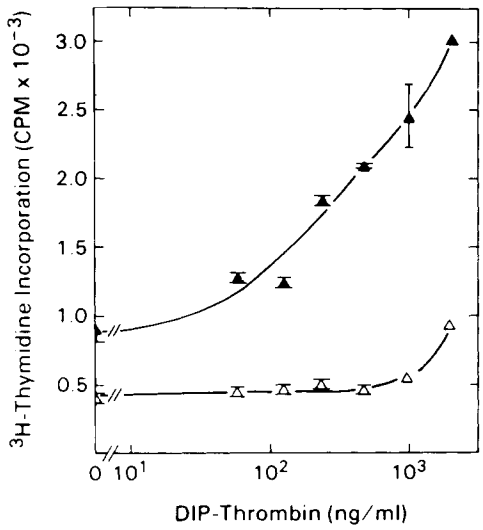

Fig. 5. Effect of DIP-α-thrombin concentration on stimulation of thymidine incorporation by γ-thrombin. Indicated concentrations of DIP-α-thrombin were added to quiescent serum-free cultures of ME cells alone (△) or in the presence of 0.5 μg/ml of γ-thrombin (▲). Thymidine incorporation was determined 24 hr later as described in Methods.

As shown in Figure 6, if either γ-thrombin or DIP-α-thrombin was initially added to quiescent ME cells and the second derivative was added up to 4 hr later, stimulation of DNA synthesis, measured by thymidine incorporation at 23 hr, was equivalent to that seen when both derivatives were added at the same time. Even 6 and 8 hr after the initial addition, addition of the second thrombin derivative caused approximately a 100% and 80% stimulation, respectively, over the first derivative alone. This suggested that the actions of these derivatives might be temporally separable by up to 8 hr. However, since DNA synthesis in these experiments was measured at one time point, it was possible that the derivatives remained active for up 8 hr but that they did not begin to initiate early events until both derivatives were present. This was a major concern in the case of DIP-α-thrombin where DIP-α-thrombin could bind to the high-affinity receptors and remain on the cell surface [7,8].

To determine if early events were initiated by addition of a single derivative, DIP-α-thrombin was added 6 hr before γ-thrombin and thymidine incorporation was analyzed to see if the early addition would shift the peak of DNA synthesis. As shown in Figure 7, DIP-α-thrombin addition prior to γ-thrombin shifts the thymidine incorporation curve by approximately 6 hr. It should be noted that this curve corresponds to and is approximately 90% as high as the curve generated if both DIP- and γ-thrombin were added at the same time (data not shown). Thus, it appears that receptor occupancy by DIP-α-thrombin alone can initiate early events.

The previous experiments (Fig. 6) suggested that γ-thrombin also initiated early events prior to DIP-α-thrombin addition. This effect of γ-thrombin is somewhat difficult to establish in experiments similar to that shown in Figure 7 because DIP-α-thrombin, even at high concentrations by itself, does not initiate DNA synthesis in the majority of these cells. Therefore, we decided to determine if γ- and DIP-α-

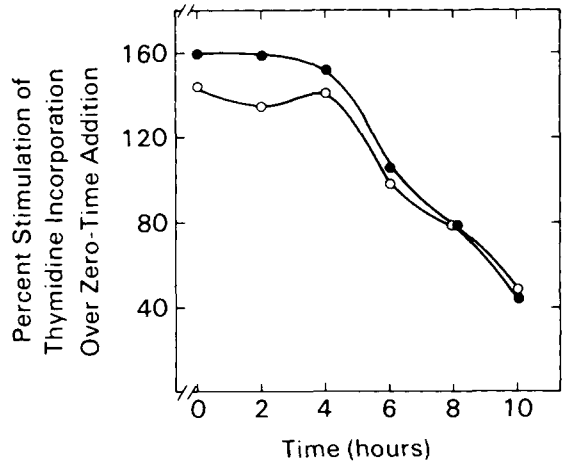

Fig. 6. Temporal separation of the stimulatory effects of γ- and DIP-α-thrombin. DIP-α-thrombin (2 μg/ml) or γ-thrombin (0.5 μg/ml) was added to quiescent serum-free cultures of ME cells at 0 time for a control level value. At the indicated times thereafter, 0.5 μg/ml of γ-thrombin (●) was added to cultures originally receiving DIP-α-thrombin, and 2.0 μg/ml of DIP-α-thrombin (○) was added to cultures originally receiving γ-thrombin. Stimulation is expressed as percentage increase over the total CPM of thymidine incorporated into cultures receiving only the initial addition of DIP-α-thrombin or γ-thrombin.

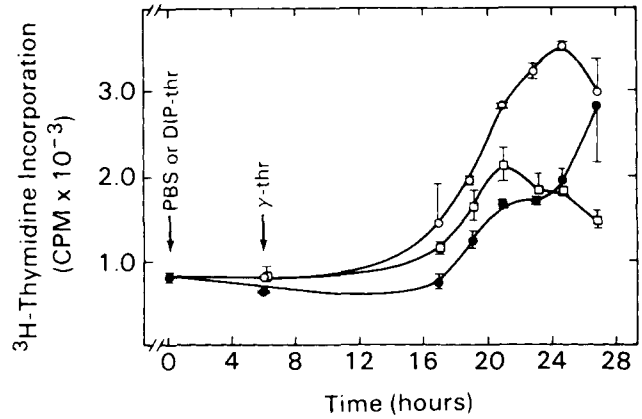

Fig. 7. Effect of DIP-α-thrombin preincubation on γ-thrombin-stimulated thymidine incorporation into ME cells. Control PBS (●) or 2 μg/ml of DIP-α-thrombin (○ and □) was added to quiescent ME cell cultures. Six hours later 1.0 μg/ml of γ-thrombin was added (○ and ●). Note: (□) represents initial addition of DIP-α-thrombin alone. Thymidine incorporation was determined at the indicated times after the initial addition using 30-min incubations with [^{3}H]thymidine as described in Methods.

thrombin could both initiate early events which could shift the thymidine incorporation curve generated by addition of native α-thrombin.

As shown in Figure 8A and B, addition of either DIP-α-thrombin or γ-thrombin 6 hr prior to native α-thrombin results in a shifting of the α-thrombin thymidine incorporation curve forward by 3–4 hr. This shift in the initiation of DNA synthesis appeared to be most pronounced by the early addition of γ-thrombin (Fig. 8B). These

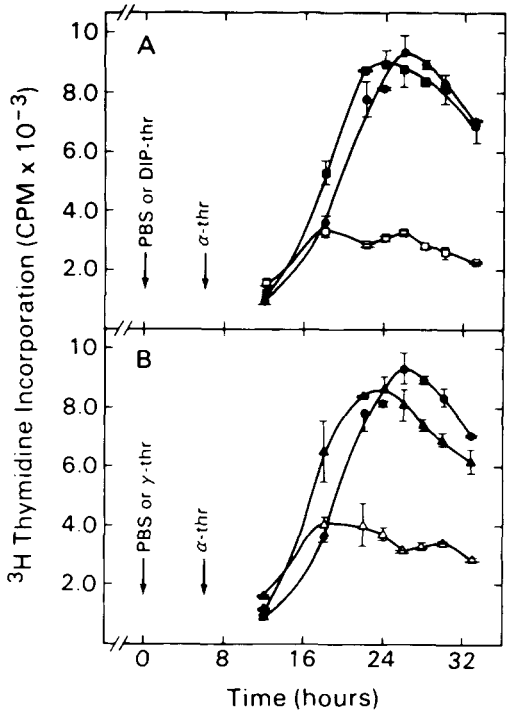

Fig. 8 Effect of DIP-α-thrombin or γ-thrombin pretreatment on native α-thrombin-stimulated thymidine incorporation. Control PBS (●) or DIP-α-thrombin at 2 μg/ml (■ and □, panel A) or γ-thrombin at 1 μg/ml (▲ and △, panel B) was added to quiescent ME cell cultures at 0 time. Six hours later native α-thrombin (2.0 μg/ml) was added (●, ■, and ▲). At indicated times after the initial addition, thymidine incorporation was determined using a 30-min incubation with [^{3}H]thymidine as described. It should be noted that early addition of γ- or DIP-α-thrombin resulted in an earlier increase and decrease in the [^{3}H]thymidine incorporation time course, suggesting a 3- to 4-hr shift in the entry of these cells into a proliferative cycle. Similar 3- to 4-hr shifts were observed in three separate experiments.

results indicate that two separate types of thrombin activities are required to initiate cell-proliferative events and that these activities each initiate a sequence of events which act like early events shortening the length of time necessary for cells to enter a proliferative cycle.

DISCUSSION

Previous studies have demonstrated that binding of proteolytically inhibited DIP-α-thrombin to high-affinity α-thrombin receptors on fibroblastic cells from mouse, chick, or hamster is not sufficient to initiate cell proliferation [6,20,23]. These studies therefore concluded that initiation requires enzymic activity and perhaps the proteolytic cleavage of either the receptor or some adjacent molecule. Derivative forms of thrombin such as γ-thrombin which are esterolytically active but which do not bind to the high-affinity α-thrombin sites also do not initiate proliferation [20,23]. We have recently shown that these enzymically active derivatives can stimulate both early and late ion fluxes which accompany initiation of cell proliferation by thrombin and other growth factors [23]. These results suggested that two separate types of

signals might be required for thrombin to initiate cell proliferation—one involving events generated by high-affinity receptor binding, the other involving enzymic activity or the subsequent stimulation of ion transport. We now report that adding γ- and DIP-α-thrombin together generates a complete signal and results in initiation of DNA synthesis and cell proliferation to levels comparable to those achieved with native α-thrombin.

Consistent with our previous studies, neither DIP-α-thrombin nor γ-thrombin alone initiated DNA synthesis or cell proliferation to levels comparable to that of α-thrombin. In contrast, if DIP-α-thrombin was added at a concentration where the high-affinity α-thrombin receptors were occupied, γ-thrombin addition to the same cells resulted in a stimulation of thymidine incorporation which approached that of the maximal stimulation by native α-thrombin. The combination of DIP-α- and γ-thrombin also caused an 80% increase in cell number over 48 hr. Thus the stimulation of thymidine by these derivatives appears to represent a true initiation of DNA synthesis and other events necessary for cells to complete a proliferative cycle.

Two lines of evidence demonstrated that the combinatory effect of γ- and DIP-α-thrombin could not be due to any residual fully active α-thrombin present in the derivative preparations. First, from the amount of fibrinolytic activity remaining in the DIP-α-thrombin preparation we would estimate a maximum of approximately 0.01% active thrombin. In our typical experiments this would correspond to approximately 0.2 ng/ml, yet addition of 15 ng/ml of α-thrombin (a 75-fold excess over the estimated amount) together with γ-thrombin could not duplicate the stimulation observed with the combination of γ- and DIP-α-thrombin. Second, addition of antithrombin III at concentrations that completely inhibited stimulation by up to 250 ng/ml of α-thrombin only partially inhibited the initiation of DNA synthesis by the combination of γ- and DIP-α-thrombin. Thus, the stimulation of DNA synthesis by the combination of γ- and DIP-α-thrombin cannot be explained by the presence of low concentrations of residual α-thrombin or by liberation of active thrombin during incubation.

The apparent requirement for receptor occupancy in initiation of cell proliferation by thrombin has become somewhat controversial. Our early studies indicated that receptor occupancy was necessary for thrombin to initiate cell proliferation [5]. This conclusion was largely based on the observation that low concentrations of serum appeared to selectively block both thrombin high-affinity binding and initiation of cell proliferation. Recent studies with thrombin activation of endothelial cells [29] and initiation of cell proliferation [22] have shown that saturation of the high-affinity receptors with DIP-thrombin does not inhibit activation or initiation by α-thrombin. Such studies might suggest that high-affinity binding is not required for α-thrombin stimulation. Our present results indicate that receptor occupancy is required as part of the signal for initiation of cell proliferation. Since occupancy of these receptors by DIP-α-thrombin is sufficient to complete an initiation signal in the presence of enzymically active γ-thrombin, it would appear that the part of the signal generated by receptor occupancy can be generated by either DIP- or α-thrombin binding to these receptors. Thus, saturating the high-affinity sites with DIP-α-thrombin would not inhibit initiation by α-thrombin, but would make the stimulation dependent only on active thrombin interaction with lower-affinity sites.

The esterase activity of thrombin derivatives appears to correlate with stimulation of ion fluxes. In previous studies, we found that two enzymically active thrombin

derivatives, nitrothrombin and γ-thrombin, stimulated Na^+, K^+-ATPase-dependent $^{86}Rb^+$ influx within 5 min after their addition to hamster fibroblasts and caused changes that also resulted in increased $^{86}Rb^+$ influx measurable 6 hr later [23]. In contrast, DIP-α-thrombin did not stimulate ion fluxes at either time. Other investigators have suggested that growth factor activation of amiloride-sensitive ^+Na influx through the $^+Na/^+H$ antiport is responsible for initiating a series of events which includes activation of the Na^+, K^+-ATPase, increasing intracellular pH, increasing Ca^{++} ion concentration, and ultimately initiating cell proliferation [30–32]. Our early studies on α-thrombin stimulation of amiloride-sensitive Na^+ uptake indicated that the initial increase of Na^+ influx was neither necessary nor sufficient to initiate DNA synthesis, but that a later amiloride-sensitive event occurring 6–8 hr after thrombin addition was necessary [18]. Since both γ-thrombin and nitro-α-thrombin, which are not mitogenic, stimulated these later ion fluxes, these fluxes by themselves are not sufficient to initiate DNA synthesis. The present results, however, suggest that these transport changes or other changes induced by enzymic activity may be combined with receptor occupancy to generate a complete signal for initiation of DNA synthesis and cell division.

Other systems have also been reported to require two separate types of cellular interaction for generating a complete signal. For example, β-transforming growth factors are only active when high-affinity epidermal growth factor (EGF) receptors are occupied by EGF or sarcoma growth factors [33]. In other studies, simultaneous addition of insulin and EGF, neither of which alone initiates SL_1 cells, resulted in stimulation of DNA synthesis [34]. Thus, EGF and insulin appear to act cooperatively to trigger the cascade of subsequent events. In similar studies, insulin or low concentrations of EGF stimulated early events which allowed later addition of EGF to initiate DNA synthesis with a shorter lag time [35]. These results indicate that some events may be required early to act as competence factors, while others are required later to allow the cells to progress into the cell cycle.

In the case of thrombin, it has been shown that initiation of DNA synthesis in hamster cells requires continued exposure of the cells to thrombin for at least 6 hr [15]. Since our results indicate that thrombin initiation requires two separate types of cell interaction, we attempted to determine if these events could be separated temporally and if one event necessarily preceded the other. In these experiments, we found that both γ- and DIP-α-thrombin could initiate early events that shortened the lag period for initiation of DNA synthesis by native α-thrombin. Moreover, combinations of γ- and DIP-α-thrombin added 4 hr apart were equally as effective as simultaneous addition independent of which derivative was added first. These results indicate that the two actions of thrombin can be physically and temporally separated but that either one is capable of independently initiating early events which move the cells forward toward entry into a proliferative state.

The exact nature of the cascade of molecular events initiated by γ-thrombin and DIP-α-thrombin remains to be elucidated. In the present studies as well as other studies with these derivatives, neither γ- nor DIP-α-thrombin alone initiated DNA synthesis to levels that approached the stimulation seen by α-thrombin alone. At high concentrations of these derivatives, however, there was some stimulation (10–30% that of the combined derivatives), suggesting that some ME cells may be partially activated in their quiescent state so that by stimulating the second set of events these cells are initiated. In the general population, however, both types of signals appear

necessary. Since ion fluxes are stimulated by enzymically active γ-thrombin but not by DIP-α-thrombin occupancy of the high-affinity binding sites, it is tempting to assume that ion fluxes play a major role in the action of α-thrombin. Subsequent to such ion fluxes, cytoskeletal changes may occur which are also required. For example, we have previously shown that microtubule stabilization by taxol inhibits initiation of DNA synthesis by α-thrombin or EGF, even when taxol is added up to 8 hr after the growth factors [19]. Microtubule disruption enhances initiation of DNA synthesis by EGF [36] and by agents which increase intracellular cAMP [37]. Thus, enzymically active γ-thrombin might initiate ion fluxes and cytoskeletal changes whereas receptor occupancy by DIP-α-thrombin activates a completely different set of metabolic effects.

In summary, α-thrombin stimulation of mitogenesis appears to require a "double-lock" mechanism. DIP-α-thrombin binds high-affinity receptors in a manner similar to the native enzyme. This binding or occupancy of these receptors generates one signal which appears to involve a nonenzymic or "hormonal" activity of thrombin. This signal could result from high-affinity protein interactions inducing protein conformational rearrangements. Low-affinity interaction of enzymically active α- or γ-thrombin generates a separate signal which requires enzymic activity and may relate to a lower-affinity proteolytic cleavage of the receptor or some other molecule on the cell surface. It would thus appear that native α-thrombin must generate both types of signals to initiate cell proliferation.

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