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# Thrombin immobilized to extracellular matrix is a potent mitogen for vascular smooth muscle cells: nonenzymatic mode of action

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**Esterolytically inactive diisopropyl fluorophosphate-conjugated thrombin (DIP-α-thrombin) stimulated $^3$H-thymidine incorporation and proliferation of growth-arrested vascular smooth muscle cells (SMCs), similar to native α-thrombin. Half-maximal mitogenic response of SMCs was obtained at 1 nM thrombin and was specifically blocked by the leech-derived, high-affinity thrombin inhibitor, hirudin. Native thrombin and a variety of thrombin species that were chemically modified to alter thrombin procoagulant or esterolytic functions were found to induce $^3$H-thymidine incorporation to a similar extent. Exposure of SMCs to DIP-α-thrombin caused a rapid and transient expression of the *c-fos* protooncogene, determined by Northern blot analysis. These results indicate that thrombin is a potent mitogen for SMCs through a distinct nonenzymatic domain. Binding of $^{125}$I-α-thrombin to SMC cultures revealed an apparent dissociation constant of 6 nM and an estimated $5.4 \times 10^5$ binding sites per cell. This binding was inhibited to the same extent by native thrombin and by its nonenzymatic form, DIP-α-thrombin. Moreover, the chemotactic fragment of thrombin (CB67-129), which failed to elicit a mitogenic response, competed for $^{125}$I-α-thrombin binding to SMCs. Cross-linking analysis of $^{125}$I-α-thrombin to SMCs revealed a specific cell-surface binding site 55 kDa in size. Finally, thrombin immobilized to a naturally produced extracellular matrix retained potent mitogenic activity toward SMCs. These observations lend support to the possibility that in vivo, subendothelial basement membranes sequester thrombin (as well as other bioactive molecules), which may stimulate localized and persistent growth of arterial SMCs. Thrombin may thus be involved directly in progression of atherosclerotic plaque formation.**

## Introduction

Thrombin, classically known for its central role in hemostasis, is also a potent activator regulating a variety of cellular-mediated events (Jackson and Nemerson, 1980; Bar-Shavit and Wilner, 1986a). The enzyme is generated from its zymogen prothrombin through the concerted action of several factors in the blood coagulation pathway and acts to convert fibrinogen to fibrin, the major constituent of a hemostatic plug (Jackson and Nemerson, 1980).

It has been recognized for several years that α-thrombin, the enzymatically active form of thrombin, is capable of initiating proliferation in quiescent fibroblasts (Chen and Buchanan, 1975; Carney and Cunningham, 1978) and macrophage-like cells (Bar-Shavit *et al.*, 1986a). We have recently demonstrated that thrombin can be immobilized onto the subendothelial extracellular matrix (ECM) in a manner that leaves the molecule functionally active and protected from inactivation by antithrombin III (AT III) (Bar-Shavit *et al.*, 1989). Therefore, under certain conditions, thrombin may be present within the vessel wall exhibiting prolonged and localized activities.

[1] AT III, antithrombin III; bFGF, basic FGF; BSA, bovine serum albumin; DIP-α-thrombin, diisopropylfluorophosphate-α-thrombin; DMEM, Dulbecco's modified Eagle's medium; DSS, disuccinimidyl substrate; ECM, extracellular matrix; EDC, (1-ethyl-3-[3 dimethylaminopropyl] carbodiimide)HCl; FCS, fetal calf serum; FGF, fibroblast growth factor; IL-1, interleukin-1; $MeSO_2$, methylsulfonyl fluoride; NPGB, p-nitrophenyl-p-guanidinobenzoate; PBS, phosphate-bufferedsaline;PDGF,platelet-derivedgrowthfactor;PPACK, D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl-ketone; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SMC, smooth muscle cell; TCA, trichloroacetic acid; TLCK, *N*-α-tosyl-L-lysylchloromethyl-ketone.

Defining factors that regulate proliferation of cells in the vessel wall provides a continuous challenge. The vascular system is composed of the intimal endothelial cells, lining the lumen and the underlying medial smooth muscle cells (SMCs), both coexisting in a quiescent growth state (Castellot *et al.*, 1982; Ross, 1986). Unregulated proliferation of the medial SMCs may thus have significant implications in the progression of arterial-wall diseases such as atherosclerosis (Ross *et al.*, 1986). Accumulating evidence has indicated that SMC proliferation commences before platelet adherence at sites of lesion and may proceed under conditions where the endothelial lumen remains intact (Ross, 1986). Therefore, initiation of atherogenesis in vivo does not necessarily require products released from platelets external to the artery and may, in fact, occur within the intact vessel wall via intrinsic mediators. Other factors, released mainly from mononuclear phagocytes, have been described as playing a key role in the progression of vascular diseases. Among these are platelet-derived growth factor (PDGF)-like molecules (DiCorleto and Bowen-Pope, 1983; Shimokado *et al.*, 1985; Martinet *et al.*, 1986), fibroblast growth factor (FGF) (Baird *et al.*, 1985; Klagsbrun and Edelman, 1989), and the multipotent mediator interleukin 1 (IL-1) (Libby *et al.*, 1988). Because under certain circumstances thrombin may be present within the vessel wall, firmly bound to the subendothelial basement membrane (Bar-Shavit *et al.*, 1989), we investigated whether it can also serve as a growth-promoting factor of vascular SMCs. Our data indicate that nonenzymatic thrombin is mitogenic for vascular SMCs, either when present in solution or when sequestered onto the subendothelial ECM. In its bound form thrombin may yield a more localized and persistent stimulation of SMC proliferation compared with its activity in the fluid phase.

## Results

### *Stimulation of arterial SMC proliferation by α-thrombin and DIP-α-thrombin*

Addition of either native α-thrombin or esterolytically inactive form of diisopropyl fluorophosphate-conjugated thrombin (DIP-conjugated α-thrombin) to quiescent SMCs resulted in stimulation of DNA synthesis (Figure 1). This finding is in contrast to the mitogenic effect of thrombin on human (or bovine, data not shown) skin fibroblasts, where only the enzymatically active thrombin was capable of inducing cell proliferation (Figure 1). The mitogenic effect of thrombin on SMCs was observed over a wide range of concentrations (0.1 nM–1 μM), with half-maximal response at 1 nM. Both forms of thrombin elicited the same degree of response in SMCs, as indicated by a five- to sixfold stimulation of $^3$H-thymidine incorporation, measured 48 h after exposure to the mitogen. Because thymidine incorporation alone may not be a sufficient index for cellular proliferation, we also measured the effect of thrombin on cell number. As indicated in Figure 2, both α-thrombin and DIP-α-thrombin induced a two- to fourfold increase in cell number over a period of 1–5 d.

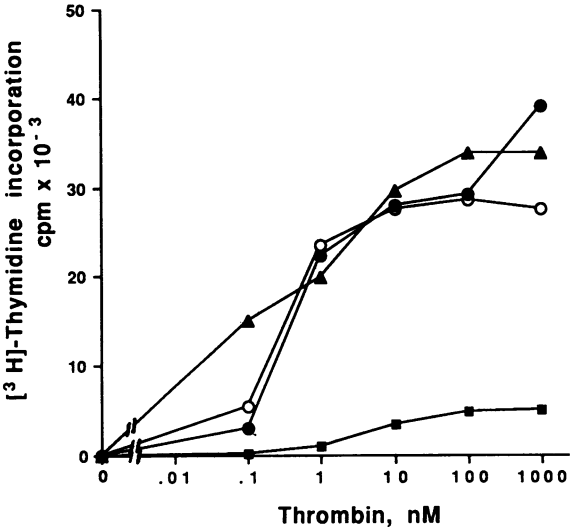


***Figure 1.*** **$^3$H-thymidine incorporation by thrombin-treated SMCs and human foreskin fibroblasts (HF).** SMCs (●; ▲) or HF (○; ■) were arrested by 48 h incubation in medium containing 0.2% FCS and stimulated with either α-thrombin (●; ○) or DIP-α-thrombin (▲; ■) for an additional 48 h in the presence of 1 μCi/ml $^3$H-thymidine. $^3$H-thymidine incorporation into TCA-insoluble material was determined. The basal incorporation of thymidine by arrested cells was 2000 ± 120 (SE) cpm per well.

Cells in S phase were visualized by autoradiography of $^3$H-thymidine–labeled nuclei. Cells arrested at $G_0/G_1$ phase were incubated either with α-thrombin, DIP-α-thrombin, or basic FGF (bFGF) in the presence of 5 μCi/ml $^3$H-thymidine for 24 h. At this time, plates were washed, fixed, and processed for autoradiography. The data revealed that 65%–80% of the nuclei were labeled after incubation with either α-thrombin, DIP-α-thrombin, or bFGF, whereas arrested cells exhibited <5% labeled nuclei (Figure 3). These results indicate that, under our experimental conditions, the majority of the cells responded to α-thrombin or DIP-α-thrombin and progressed to S phase.

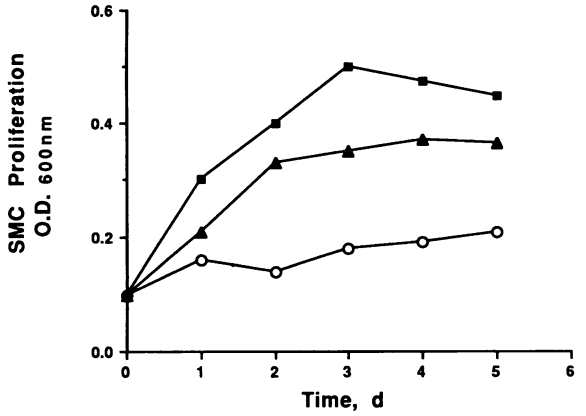


***Figure 2.*** **Effect of α-thrombin and DIP-α-thrombin on the rate of SMC proliferation.** SMCs ($5 \times 10^3$ cells/well) were seeded into wells of 96-well plates in medium containing 10% FCS. Twenty four hours after seeding, the medium was replaced by medium containing 0.2% FCS, and cultures were incubated for 48 h. α-Thrombin (1 μM, ■) or DIP-α-thrombin (1 μM ▲) was then added to some of the wells, and the cell number in the presence (■; ▲) and absence (○) of thrombin was evaluated by determination of methylen blue uptake, as described in Materials and methods.

### *Induction of SMC proliferation by modified thrombin preparations*

Selectively modified thrombin preparations were examined to determine whether alterations in the procoagulant exosite, or in the catalytic site, affect the mitogenic activity of thrombin in SMCs. As demonstrated in Figure 4, the nonclotting forms γ-thrombin and $NO_2$-α-thrombin elicited $^3$H-thymidine incorporation by SMCs to an extent that was ~80% of that induced by native thrombin. Moreover, serine- or histidine-blocked thrombin forms (i.e., methylsulfonyl fluoride [$MeSO_2$]- and *N*-α-tosyl-L-lysylchloromethyl-ketone [TLCK]-α-thrombin, respectively) induced a mitogenic response similar to that exerted by DIP-α-thrombin (Figure 4). The same level of induction was obtained by catalytically inactive D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl-ketone (PPACK)-α-thrombin subjected to fibrinopeptide exosite affinity labeling. These results clearly indicate that thrombin stimulation of DNA synthesis in SMCs is mediated largely through a nonproteolytic domain and cannot be related to a residual esterolytic activity occasionally found in DIP-α-thrombin preparations. However, as indicated in Fig. 4, the chemotactic peptide CB67-129 (derived from thrombin) and the macrophage mitogenic "loop B" peptide (Bar-Shavit *et al.*, 1986a; Bar-Shavit and Wilner, 1986a,b), failed to elicit incorporation of $^3$H-thymidine in quiescent SMCs.

The specificity of thrombin-induced SMC proliferation was demonstrated by selective blocking of $^3$H-thymidine incorporation in the presence of hirudin. This leech-derived thrombin inhibitor inhibited proliferation of those SMCs induced by DIP-α-thrombin, but not those induced by serum (Figure 5). The fact that hirudin-blocked thrombin induced mitogenicity in SMCs indicates that the mitogenic domain for SMCs may reside in the vicinity of thrombin' cell interaction exosite, described for macrophages by Bar-Shavit *et al.* (1986a).

### *DIP-α-thrombin induces a rapid and transient expression of c-fos protooncogene in arterial SMCs*

Addition of growth factors (i.e., serum, PDGF, IL-1) to fibroblasts or SMCs rapidly induces mRNA that encodes the *c-fos* protooncogene (Libby *et al.*, 1988). The product of this gene is a nuclear-associated protein that may be involved in signaling the early events in commitment of cellular division (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984; Kindy and Sonenshein, 1986). Because thrombin was found to stimulate SMC DNA synthesis in a nonenzymatic fashion, we tested the effect of DIP-α-thrombin on *c-fos* protooncogene transcript levels. Northern blot analysis revealed that RNA isolated from DIP-α-thrombin–treated SMC cultures contained elevated levels of *c-fos* transcript compared with non-stimulated cells (Figure 6). This increase reached maximal levels after 30 min incubation with DIP-α-thrombin, decreased back to control levels by 90 min, and remained unchanged thereafter, up to 4 h. Similar effects on the expression of *c-fos* mRNA were observed with PDGF and other growth factors, demonstrating that DIP-α-thrombin induces early events typical to growth-committed cells.

### *Binding of $^{125}$I-α-thrombin to SMCs*

Thrombin induction of SMC proliferation suggests the existence of specific receptors on the surface of these cells. Therefore, experiments were performed to characterize such receptor(s) in terms of binding affinity, binding capacity, and time required for obtaining maximal $^{125}$I-α-thrombin binding to SMCs. As demonstrated in Figure 7, an apparent saturation of $^{125}$I-α-thrombin binding was achieved at a concentration of 15 nM. Nonspecific thrombin binding did not exceed 20% of total binding, as determined in the presence of 1000-fold excess unlabeled thrombin. Scatchard analysis of the

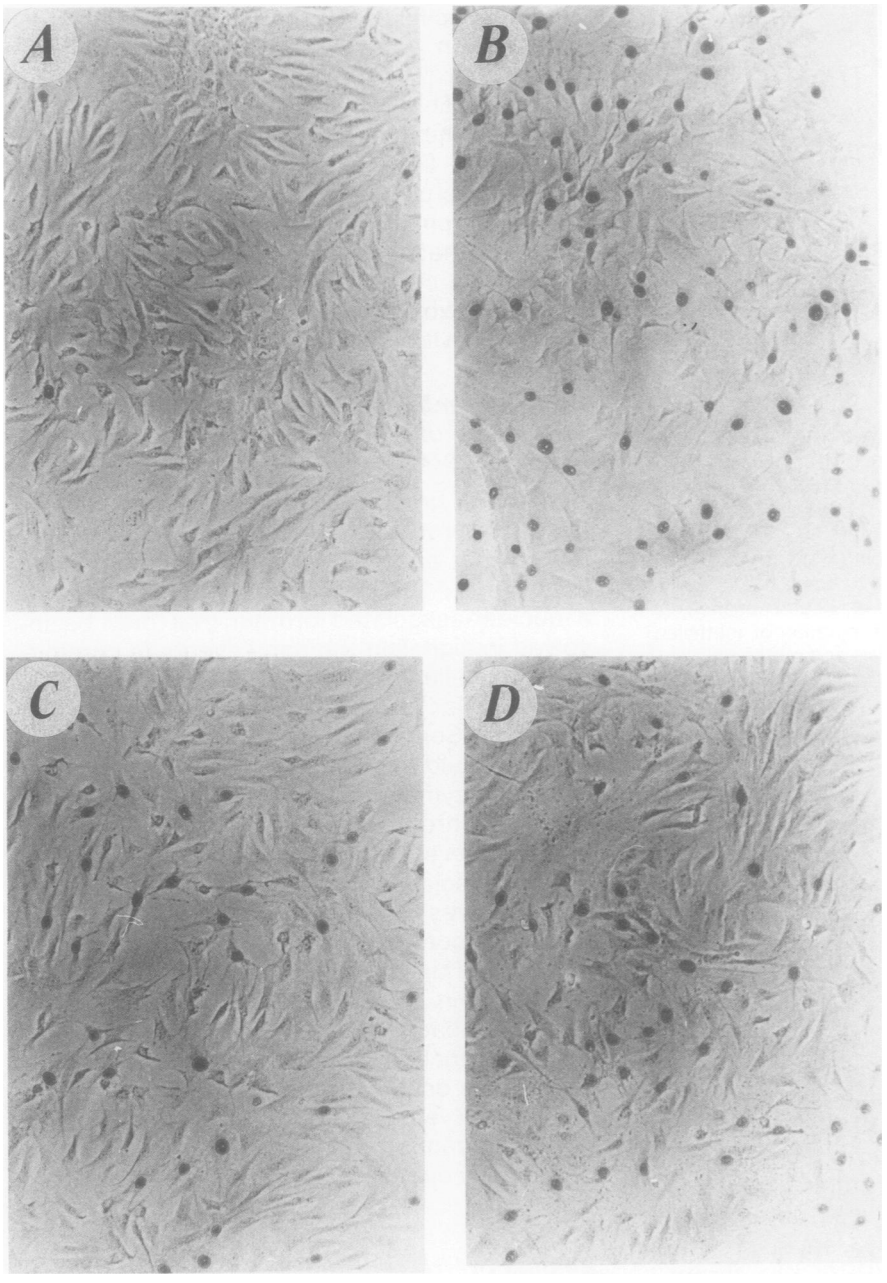


***Figure 3.*** **Autoradiography of labeled nuclei.** Quiescent SMCs maintained in medium containing 0.2% FCS were incubated with (A) 0.2% FCS, (B) 1 $\mu$M $\alpha$-thrombin, (C) 1 $\mu$M DIP-$\alpha$-thrombin, and (D) 10 ng/ml bFGF, in the presence of 5 $\mu$Ci/ml $^{3}$H-thymidine for 24 h. The cultures were then washed extensively with PBS, fixed with methanol, and dried. Autoradiography of labeled nuclei was performed as described in Materials and methods.

data revealed a single class of binding sites over the concentration range of thrombin studied, with estimated 540 000 binding sites per cell (Figure 7, insert). The apparent dissociation constant of thrombin interaction with SMCs was $6.3 \times 10^{-9}$ M, similar to that observed with fibroblasts (Glenn *et al.*, 1980; Perdue *et al.*, 1981) or macrophages ($7.5 \times 10^{-9}$M) (Bar-Shavit *et al.*, 1986b; Kindy and Sonenshein, 1986; Ross, 1986). Competition studies with either native $\alpha$-thrombin or DIP-$\alpha$-thrombin revealed the same inhibition curve of $^{125}$I-$\alpha$-thrombin binding to SMCs over a wide range of concentration (0.05–100 $\mu$g/ml) (Figure 8). Fifty percent inhibition was observed at ~28 nM (~0.95 $\mu$g/ml) of both native and DIP-$\alpha$-thrombin. This result indicates that both native and DIP-$\alpha$-thrombin share common cell-surface receptor sites. Moreover, the chemotactic peptide CB67-129 inhibited the binding of $^{125}$I-$\alpha$-thrombin by 76% at 13.4 $\mu$M and by 57.5% at 6.7 $\mu$M (Figure 8, insert). As indicated in Figure 8 (insert, lanes D and E), the macrophage mitogenic peptide, "Loop B," failed to compete with $^{125}$I-$\alpha$-thrombin binding

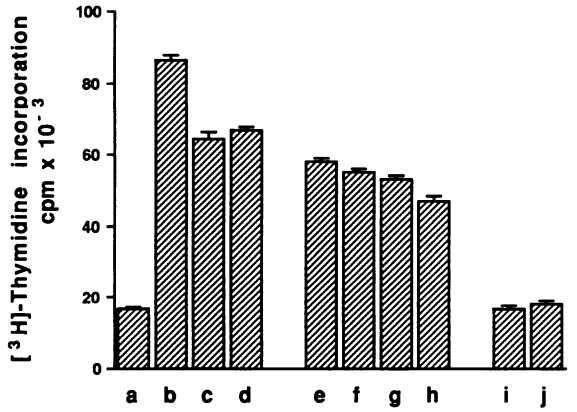


***Figure 4.*** **Effect of various chemically modified thrombin preparations and fragments on SMC proliferation.** Arrested SMCs (a) were incubated for 48 h with 1 $\mu$M of (b) native $\alpha$-thrombin, (c) $\gamma$-thrombin, (d) $NO_2$-$\alpha$-thrombin, (e) DIP-$\alpha$-thrombin, (f) TLCK-$\alpha$-thrombin, (g) $MeSO_2$-$\alpha$-thrombin, or with (h) catalytically blocked fibrinopeptide exosite affinity-labeled thrombin. The effect of (i) the chemotactic peptide CB 67-129 and (j) the macrophage mitogenic synthetic peptide "Loop B" was also analyzed. Determination of $^3$H-thymidine incorporation was performed as described in Materials and methods.

to SMCs. We conclude that, although the chemotactic peptide CB67-129 is not capable of promoting vascular SMC proliferation, it shares common cell-surface binding sites with $\alpha$-thrombin. The synthetic "Loop B" peptide, however, did not compete for $^{125}$I-$\alpha$-thrombin binding to the putative thrombin receptor and hence did not promote cell proliferation.

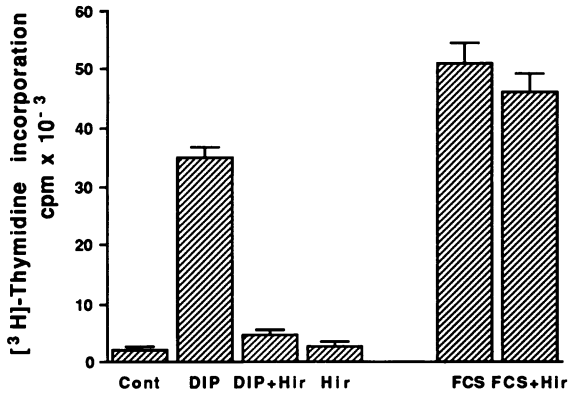


***Figure 5.*** **Selective inhibition of thrombin-induced mitogenicity with hirudin.** Quiescent SMCs were incubated with DIP-$\alpha$-thrombin (1 $\mu$M) in the absence (DIP) and presence (DIP-Hir) of hirudin (1:1 ratio), or with 10% FCS in the absence (FCS) or presence (FCS-Hir) of hirudin. Cells were also incubated in the absence of added factors (Cont) and presence of hirudin alone (Hir). Incorporation of $^3$H-thymidine was measured as described in Materials and methods.

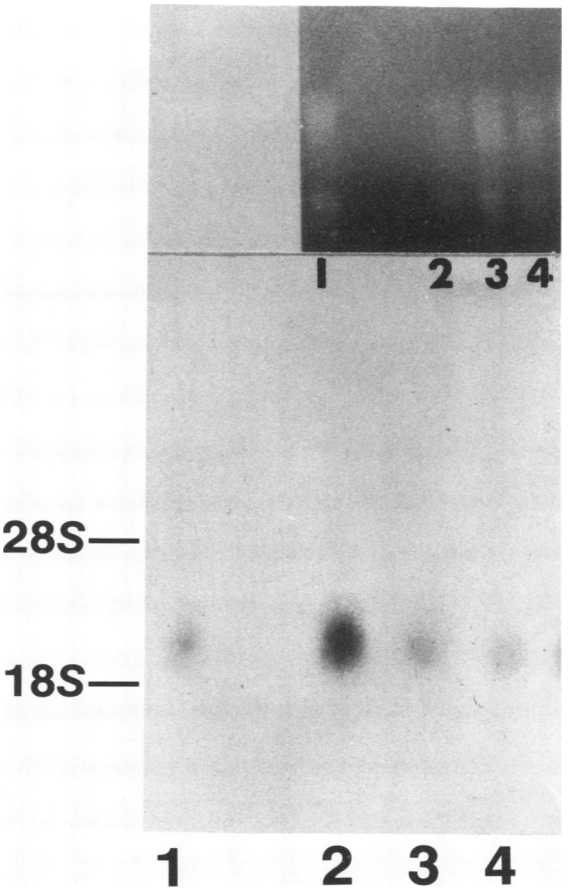


***Figure 6.*** **DIP-$\alpha$-thrombin–mediated transient induction of *c-fos* mRNA in SMCs.** Quiescent SMCs were stimulated with DIP-$\alpha$-thrombin ($10^{-6}$M) for the indicated periods of times. At the end of each incubation period, plates were washed with cold PBS and dissolved in guanidinium thiocyanate buffer for RNA isolation. Northern blot analysis and hybridization were performed as described in Materials and methods. Lanes: (1) quiescent SMCs at $G_0/G_1$ phase; (2) 30 min; (3) 2h; and (4) 4h; stimulation with DIP-$\alpha$-thrombin. Insert: RNA samples corresponding to the above lanes separated on 1% agarose-formaldehyde gel and stained with ethidium bromide.

### *Cross-linking of $^{125}$I-$\alpha$-thrombin to cell-surface receptors*

Thrombin binding sites on the surface of SMCs were identified by the affinity cross-linking technique. For this purpose intact SMCs were incubated with increasing concentrations of the bifunctional cross-linkers (1-ethyl-3-[3 dimethylaminopropyl] carbodiimide)HCl (EDC) (0.5 or 1 mM) or disuccinimidyl substrate (DSS) (0.5 mM). Subsequent analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and autoradiography revealed a 90-kDa, iodinated

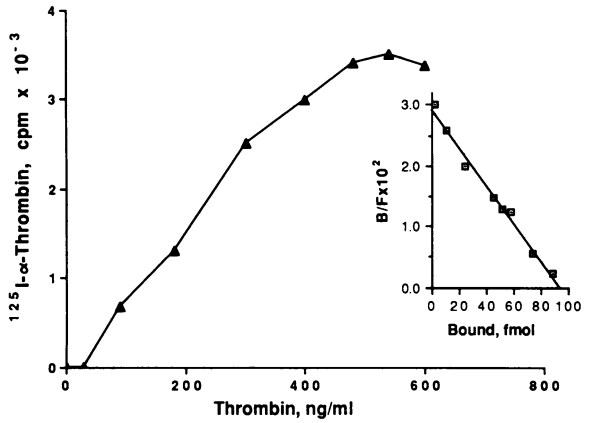


***Figure 7.* Binding of $^{125}$I-α-thrombin to monolayers of SMCs.** Increasing concentrations of $^{125}$I-α-thrombin were added to monolayers of SMCs in binding buffer containing 1% BSA. After 4 h incubation at 4°C, the cultures were washed three times with PBS and the cells dissolved in 1M NaOH. Nonspecific binding was determined in the presence of 1000-fold excess unlabeled thrombin. Insert: Scatchard plot analysis of the binding data.

component representing the product of the cross-linking reaction (Figure 9, lanes a, c, and e). This result suggests that the apparent molecular mass of thrombin receptor on SMCs is 55 kDa. Cross-linking of $^{125}$I-α-thrombin to its specific cell-surface receptors was inhibited in the presence of excess unlabeled thrombin (Figure 9, lanes b, d, and f).

### *ECM-bound thrombin is mitogenic for vascular SMCs*

It has been proposed that one of the roles of extracellular matrices in vivo is to sequester and stabilize biologically active molecules to provide a more localized and persistent stimulation of surrounding tissues (Smith *et al.*, 1982; Knudsen *et al.*, 1986). Because thrombin can be immobilized on the subendothelial ECM in a manner that leaves the molecule functionally active while protected from inhibition by circulating AT III (Bar-Shavit *et al.*, 1989), we investigated whether ECM-bound thrombin is also capable of promoting SMC proliferation. The subendothelial ECM produced by bovine vascular and corneal endothelial cells contains bFGF (Vlodavsky *et al.*, 1987), which is a potent mitogen for SMCs. Therefore, use of these matrices might mask the mitogenic effect of ECM-immobilized thrombin. Thus, ECM produced by PF-HR-9 mouse endodermal carcinoma cells which is devoid of bFGF (Rogelj *et al.*, 1989) was employed. As shown in Figure 10, PF-HR-9 ECM-bound thrombin was capable of promoting a three- to fourfold stimulation of vascular SMC proliferation, compared with cells maintained on PF-HR-9 ECM alone (which had no effect on SMC growth). Maximal stimulation was observed 3–5 d after seeding the cells in contact with ECM-bound thrombin in medium containing 0.2% fetal calf serum (FCS). The possibility that some of the ECM-immobilized α-thrombin is released during the 5 d assay for SMC proliferation was next addressed. For this purpose, release of ECM-bound $^{12}$I-α-thrombin was measured every day during 5d, in the absence and presence of $5 \times 10^4$ SMCs seeded at day 1. The results revealed that release of ECM-bound $^{125}$I-α-thrombin was twice as high in the presence than absence of cells, but was rather minimal in both cases and never exceeded 12% of the initial amount of ECM-bound thrombin. In fact, the total amount of $^{125}$I-α-thrombin released into the incubation medium was ~2 ng, corresponding to a concentration of $5 \times 10^{-11}$M. As demonstrated in Figure 1, this concentration is much lower than the $10^{-7}$M of thrombin required to induce the observed mitogenic effect. These results suggest that thrombin, when immobilized to the subendothelial basement membrane in vivo, may elicit a localized, long-acting stimulation of SMC proliferation.

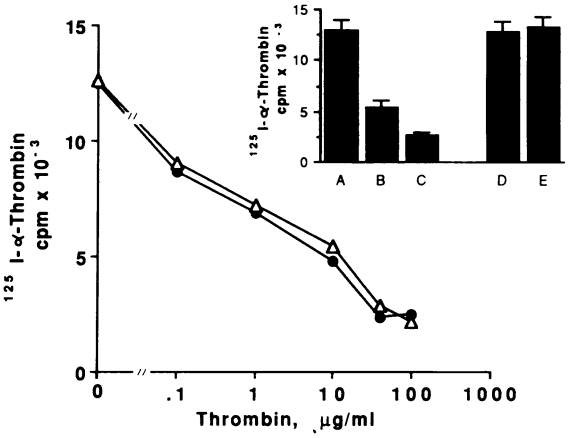


***Figure 8.* Competition of $^{125}$I-α-thrombin binding to SMCs by unlabeled α-thrombin and DIP-α-thrombin.** Confluent cultures of SMCs ($5 \times 10^5$ cells/16-mm well) were incubated (4h, 4°C) in binding medium with $^{125}$I-α-thrombin (~7nM, 200 000 cpm/well) in the presence of increasing amounts of unlabeled α-thrombin (●), or DIP-α-thrombin (△). Insert: SMC cultures were incubated (4h, 4°C) with $^{125}$I-α-thrombin (14nM) in the (A) absence and presence of (B) 50 μg/ml (6.25 μM) and (C) 100 μg/ml (13 μM) of unlabeled chemotactic peptide CB 67-129, or (D) 50 μg/ml (~30 μM) and (E) 100 μg/ml (~60 μM) of unlabeled "Loop B" peptide. The cultures were washed (3 times) with PBS and the amount of cell-bound $^{125}$I-α-thrombin measured as described in Materials and methods.

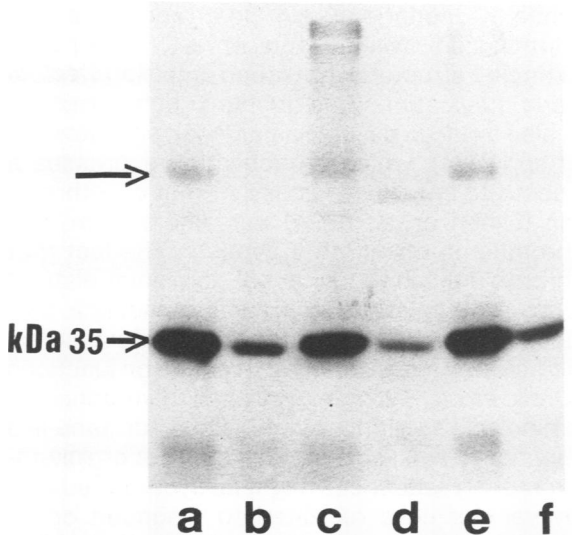


***Figure 9.*** **Cross-linking of $^{125}$I-$\alpha$-thrombin to SMC cultures.** SMCs were incubated with $^{125}$I-$\alpha$-thrombin (200 ng/ml) for 4 h at 4°C in the presence (a, c, and e) or absence (b, d, and f) of 1000-fold excess unlabeled thrombin. Cells were then extensively washed and incubated with EDC (a and b, 0.5 mM; c and d, 1 mM), or with 0.5 mM DSS (e and f) for an additional 30 min at room temperature. The cells were then solubilized in SDS sample buffer and analyzed on a 5%–15% gradient SDS-PAGE.

## Discussion

Uncontrolled proliferation of vascular SMCs contributes to the progression of arterial-wall diseases such as atherosclerosis (Ross, 1986; Ross *et al.,* 1986). The present study demonstrates that the serine protease thrombin contains a potent mitogenic activity for vascular SMCs, distinct from its catalytic site. Although its classical role as a procoagulant agent is well known, thrombin is now emerging as a growth-promoting factor. The first observation was made in fibroblasts (Chen and Buchanan, 1975), where it was demonstrated that only the intact, fully active enzyme can stimulate cells to proliferate. Unlike fibroblasts, thrombin-treated macrophage-like cells enter S phase totally independent of thrombin proteolytic site. The macrophage growth-promoting domain was found to reside in a specific cell-recognition exosite region of thrombin B-chain, separate and distinct from its proteolytic center (Bar-Shavit *et al.,* 1986a,b). Likewise, thrombin function as a selective chemotaxin toward cells of the mononuclear-phagocytic lineage is mediated through a nonenzymatic site, located at residues 338–400 of thrombin B-chain (Bar-Shavit *et al.,* 1983b; Bar-Shavit and Wilner, 1986a) and containing the "Loop B" mitogenic domain (Bar-Shavit *et al.,* 1986a).

The observed stimulation of DNA synthesis in SMCs was specific for thrombin, because complexes formed with the leech-derived polypeptide thrombin inhibitor, hirudin, were devoid of mitogenic activity. Hirudin itself had no effect on either arrested or serum-stimulated SMCs. The fact that hirudin blocked thrombin-induced $^{3}$H-thymidine incorporation may indicate that the SMC mitogenic domain resides perhaps in the vicinity of the chemotactic site. In fact, our data demonstrate that thrombin-derived chemotactic peptide competed with $^{125}$I-$\alpha$-thrombin binding to SMCs (Figure 8), although it was incapable of promoting cell division (Figure 4). Thus, it appears that thrombin binding region to SMCs receptors resides within the CB67-129 chemotactic peptide, which requires additional sequence(s) to elicit a mitogenic response. The SMC growth-promoting site in thrombin differs, however, from the macrophage mitogenic domain, because the synthetic tetradecapeptide, representing residues 367–380 of thrombin B-chain, failed to elicit any mitogenic response in growth-arrested SMCs but exhibited a potent growth-promoting activity on macrophage-like cells (Bar-Shavit *et al.,* 1986a). Delineation of SMC-specific mitogenic domain in thrombin B-chain exosite may have important physiological implications toward localization/identification of potential sequences in thrombin circulating degradation products of thrombin. Such frag-

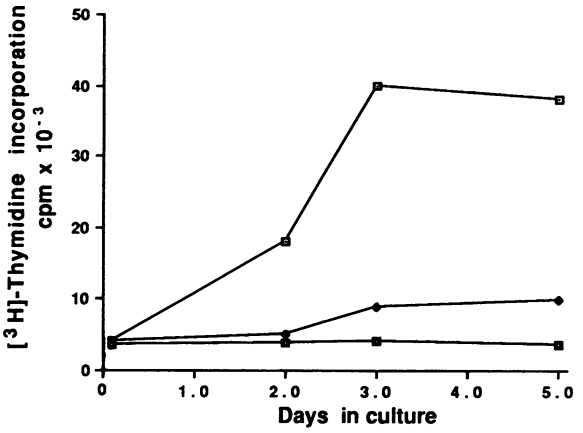


***Figure 10.*** **PF-HR-9/ECM–bound thrombin induces $^{3}$H-thymidine incorporation in SMCs.** SMCs were seeded (5 × 10$^{4}$ cells/dish) into 35-mm dishes (♦) and into dishes coated with ECM produced by PF-HR-9 cells (⊡; □). Some of the ECM-coated plates were preincubated (4h, 37°C) with 10$^{-6}$M $\alpha$-thrombin and washed free of unbound thrombin before seeding of the cells (⊡). SMCs maintained in medium containing 0.2% FCS were pulsed at various times after seeding with $^{3}$H-thymidine (1 $\mu$Ci/plate) for 24h and measured for DNA synthesis.

ments may escape inhibition by the traditional thrombin inhibitors: protease nexin (Kramer and Vogel, 1983), AT III (Rosenberg, 1977), or heparin cofactor II (Tollefsen *et al.,* 1982).

Thrombin mitogenic effect is initiated through interaction with specific cell-surface receptors on SMCs. The apparent dissociation constant of this interaction (6 nM) correlates with thrombin mitogenic activity, reaching a half-maximal effect at ~1 nM. The number of binding sites was similar to that previously found in fibroblasts and macrophage-like cells (~500 000/cell) (Perdue *et al.,* 1981; Bar-Shavit *et al.,* 1983a; Carney and Cunningham, 1978). Thrombin-specific receptors on SMCs recognize both native and nonenzymatic DIP-$\alpha$-thrombin to a similar extent and, with lower affinity, the thrombin-derived chemotactic fragment CB67-129. This cell-surface receptor appeared to be a 55-kDa protein, as indicated by its specific cross-linkage with $^{125}$I-$\alpha$-thrombin.

Thrombin is generated traditionally in response to vascular injury, leading to platelet adhesion and aggregation on the surface of the exposed subendothelium to establish a fibrin clot. Under normal conditions, however, when the integrity of the subendothelium is retained, thrombin has been shown to induce gap formation between adjacent endothelial cells in a rapid, non-cytotoxic, reversible manner (Laposata *et al.,* 1983; Garcia *et al.,* 1986). Thus, thrombin, when generated under certain circumstances on the luminal surface of the endothelium (Stern *et al.,* 1985), may pass through the endothelial cell layer and reach subendothelial structures. In addition, during the process of fibrin-clot formation, thrombin may become trapped within the fluid phase of the fibrin gel (Kaminski *et al.,* 1983; Wilner *et al.,* 1981). This thrombin can be recovered intact and active on fibrinolysis and hence may become accessible to the vascular subendothelium. Our studies on the properties and biological activity of ECM-bound thrombin demonstrate that immobilized thrombin is a potent mitogen for vascular SMCs, while being protected from inactivation by its physiological inhibitor AT III (Bar-Shavit *et al.,* 1989). Thrombin sequestered by subendothelial ECM may thus be involved in the progression of the atherosclerotic process.

Serine proteases are closely related proteins descending perhaps by splicing of a common gene ancestor (Neurath, 1984). The cDNA sequences of the human and bovine prothrombin gene have been determined (Craik *et al.,* 1982; MacGillivray and Davie, 1984). Beyond the 10 $\gamma$-carboxyglutamic acid residues, which are vitamin K-dependent and posttranscriptionally synthesized (Magnusson *et al.,* 1975), two "kringle" structures are found, implying tandem gene duplication. This double kringle structure is also found in tissue plasminogen activator (Ny *et al.,* 1984), which on activation expresses a substrate specificity similar to that of $\alpha$-thrombin (Lijnen *et al.,* 1984) but differs from prothrombin in many other aspects. The fact that thrombin exhibits functional domain(s) distinct from its catalytic site provides evidence that despite a common ancestor gene, thrombin has acquired genes inserted at exon-intron junctions (Craik *et al.,* 1982) to yield the divergence of amino acid sequences with unique hormone-like functions. It is not unlikely that such growth-promoting sequences might be synthesized also by various cells recruited to wounded or inflamed regions within the vessel wall.

The present study demonstrates nonenzymatic activity of a serine protease residing in a distinct domain separate from the proteolytic site. Under certain conditions, thrombin may be immobilized onto the vascular subendothelium, where it may express its functional activities (chemotaxis, mitogenesis) and yield a considerably prolonged and localized mitogenic stimulation of SMCs, thus directly contributing to the progression of atherosclerosis.

## Materials and methods

### *Cells*

SMCs were isolated from the bovine aortic media as previously described (Castellot *et al.,* 1982). Briefly, the abdominal segment of the aorta was removed and the fascia cleaned away under a dissecting microscope. The aorta was cut longitudinally, and small pieces of the media were carefully stripped from the vessel wall. Two or three such strips with average dimensions of 2 × 2 mm were placed in 60-mm-diam tissue culture dishes (Falcon Labware Division, Becton Dickinson, Oxnard, CA) that contained Dulbecco's modified Eagle's medium (DMEM; 1 g glucose/l) supplemented with 10% FCS, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (GIBCO, Grand Island, NY). Within 7–14 d, large patches of multilayered cells had migrated from the explants. Approximately 1 wk later, SMCs from a 60-mm-diam tissue culture dish were subcultured into a 100-mm tissue culture plate (~4–6 × $10^5$ cells/plate). The cultures (passage 3-8) exhibited typical morphological characteristics of vascular SMCs, and the cells were specifically stained with monoclonal antibodies that selectively recognize muscle form of actin (HHF-35). This antibody does not recognize endothelial cells or fibroblasts (Tsukada *et al.,* 1987). Foreskin fibroblasts (passage 3-9) were maintained in DMEM (1 g glucose/l) supplemented with 10% FCS and antibiotics as described above. PF-HR-9 cells derived from a differentiated mouse endodermal carcinoma (Chung *et al.,* 1978; Rogelj *et al.,* 1989) were maintained in DMEM (4.5 g glucose/l) containing 10% FCS and antibiotics. Cells were maintained at 37°C in 8% $CO_2$-humidified incubators and subcultured weekly at a 1:10 split ratio after dissociation with saline

containing 0.05% trypsin, 0.01 M sodium phosphate, pH 7.2, and 0.02% EDTA (Biological Industries, Bet-Haemek, Israel).

### Preparation of dishes coated with ECM

For preparation of PF-HR-9 ECM, cells ($10^5$/35-mm dish) were seeded into tissue culture dishes precoated with fibronectin (50 μg/dish) (kindly provided by Kabi Vitrum AB, Stockholm, Sweden) to enforce a firm adhesion of the ECM to the plastic substratum. Ascorbic acid (50 μg/ml) (Sigma Chemicals, St. Louis, MO) was added on days 2 and 4. Six to seven days after seeding the cells, the ECM was exposed by dissolving (3 min at 22°C) the cell layer with a solution containing 0.5% Triton X-100 and 20 mM $NH_4OH$ in phosphate-buffered saline (PBS), pH 7.4, followed by four washes in PBS (Gospodarowicz *et al.*, 1984). Major constituents of the HR-9-ECM were laminin, entactin, collagen type IV, and heparan sulfate proteoglycans (Kramer and Vogel, 1984; Gospodarowicz *et al.*, 1984). PF-HR-9 cells were shown to produce ECM devoid of bFGF (Rogelj *et al.*, 1989). This ECM did not promote the proliferation of vascular endothelial cells and SMCs.

### Human thrombin preparations

Highly purified human α-thrombin was prepared from fraction III paste, evaluated for purity, and characterized as described previously (Fenton *et al.*, 1977a,b; Perdue *et al.*, 1981). Specific clotting activity of the α-thrombin preparation was 3975 U/mg. The preparation used in the experiments consisted of 98% α-, 1.8% β-, and 0.7% γ-thrombins and was titrated to >85% active enzyme with p-nitrophenyl-p-guanidinobenzoate (NPGB).

**γ-Thrombin.** α-Thrombin was converted essentially to γ-thrombin by controlled passage of α-thrombin through trypsin immobilized on agarose at pH 6.2. The preparation was titrated to 79% esterase activity with NPGB, and was composed of 0.4% α-, 15.2% β-, and 84.4% γ-thrombin. It possessed only 1.7 U/mg clotting activity.

**$NO_2$-α-thrombin.** α-Thrombin was nitrated as described before (Perdue *et al.*, 1981) by the use of tetranitromethane at pH 7.8. Specific activity was reduced from 3975 to 14 clotting U/mg and the NPGB-titratable active sites were reduced from 84.4% to 66%.

**DIP-α-thrombin.** α-Thrombin with a specific activity of 2800 U.S. clotting units/mg was treated repeatedly with 0.2M DIP at pH 8.0 for 30 min until clotting activity was diminished to ~0.5 U/mg and active esterolytic titrable sites were reduced to an undetectable level, as described previously (Perdue *et al.*, 1981).

**$MeSO_2$-α-thrombin.** α-Thrombin was treated with excess $MeSO_2$-F for 30 min at 24°C and dialyzed overnight against 0.75 M NaCl to remove unbound inhibitor. Clotting activity of $MeSO_2$-α-thrombin was reduced from 3975 U/mg to 1.2 U/mg; NPGB esterase activity was negligible.

**TLCK-α-thrombin.** α-Thrombin was alkylated by repeated treatment at pH 7.4 with an ~100-fold molar excess of TLCK over a period of 2 h until clotting activity was reduced from 3975 to 0.85 U/mg and esterase activity, estimated by titration with NPGB, was reduced from ~85% to 7%.

Preparation of the catalytically inactivated fibrinopeptide exosite affinity label D-Phe-Pro-Arg-$CH_2$-α-thrombin (PPACK-α-thrombin) was performed as described before (Fenton *et al.*, 1977a,b).

Modified thrombin preparations were dialyzed against 0.75 M NaCl and stored frozen at −70°C.

### Iodination of thrombin

Radiolabeling of thrombin was carried out by the use of the Iodogen method as described by Glenn *et al.* (1980). Briefly, Iodogen (Pierce, Rockford, IL) was dissolved at 1 mg/ml in $CH_2Cl_2$; 100 μl of this solution was placed in a glass tube (10 × 75 mm) and the Iodogen was coated on the surface by drying under a stream of $N_2$. Approximately 100 μg of α-thrombin and 1.0 mCi $Na^{125}I$ (100mCi/ml, carrier free; International Amersham, UK) were added to 0.1 M NaCl containing 50 mM sodium phosphate, pH 7.0, and 160 μM benzamidine. The mixture was added to the Iodogen-coated tube and incubated for 10 min at 4°C. Iodination was terminated by removing the reaction mixture from the Iodogen-coated tube. Free iodine and benzamidine were removed by gel filteration (Bio-Gel P-2; Bio-Rad Laboratories, Richmond, CA) and dialysis against 0.75 M NaCl buffered with 50 mM sodium phosphate, pH 7.0, at 4°C. The $^{125}I$-labeled preparations had a specific activity of 5–10 × $10^6$ cpm/μg and comigrated as a single band with unlabeled thrombin on SDS-PAGE. $^{125}I$-α-thrombin retained nearly 100% of its catalytic activity (measured by Chromozyme-TH assay, Sigma Chemicals, St. Louis, MO) and mitogenic activities.

### $^3H$-thymidine incorporation

SMCs were plated at an initial density of 4 × $10^4$ cells/well in 24-well plates (16-mm diameter) (Nunc, Roskilde, Denmark) in DMEM supplemented with 10% FCS. Twenty-four hours after seeding, medium was replaced with medium containing 0.2% heat-inactivated FCS, and, 48 h later, the cells were exposed to growth stimulants for an additional 24–48 h. DNA synthesis was assayed by measuring the radioactivity incorporated into trichloro acetic acid-insoluble material after labeling the cells with $^3H$-thymidine (1 μCi/well) (500 mCi/mmol; New England Nuclear, Boston, MA) for a period of 24 h, as described by Bar-Shavit *et al.* (1986a).

### Cell proliferation assay

SMCs were seeded and exposed to growth stimulants as described above. One to six days after seeding, the cells were fixed with 2.5% formaldehyde in PBS. The plates were immersed in a bath of 0.01M borate buffer (pH 8.5), stained (10 min at 24°C) with 0.1 ml methylen blue/well (1% in 0.1M borate buffer, pH 8.5), and washed four times in borate buffer. This procedure removed practically all non-cell-bound dye. Specific cell-incorporated methylen-blue was dissolved with 0.2 ml of 0.1N HCl (40 min at 37°C) and determined by reading the absorbance at 600 nm. Uptake of methylen blue was linearly correlated to the number of viable cells (Goldman and Bar-Shavit, 1979).

### Binding of $^{125}I$-α-thrombin to SMCs

Cells were grown to confluency in 16-mm culture wells (5 × $10^5$ cells/well). Confluent cultures were transferred to 4°C, washed once with PBS followed by 1 h incubation in PBS containing 1% bovine serum albumin (BSA). $^{125}I$-α-thrombin was then added at the indicated concentration, and, after 4 h incubation (except when stated otherwise), the cultures were washed three times with PBS, dissolved in 1N NaOH, and the radioactivity determined by counting in a Beckman model 5500 γ-counter. Nonspecific binding was determined in the presence of a 1000-fold excess unlabeled thrombin.

### Cross-linking of $^{125}I$-α-thrombin to SMCs

Binding of $^{125}I$-α-thrombin to SMCs was carried out as described (Glenn *et al.*, 1980). Approximately 20 nM $^{125}I$-α-

thrombin was added to confluent SMC cultures in the presence and absence of 1000-fold excess unlabeled thrombin. After 4 h incubation at 4°C, the cells were washed extensively with PBS, then incubated (30 min, 37°C, gentle mixing) with the bifunctional cross-linker EDC (increasing concentrations up to 1mM) in PBS containing 1% BSA at pH 6.5. At the end of this period, samples were washed and dissolved in SDS-polyacrylamide sample buffer for analysis on SDS-PAGE.

### *Autoradiography of labeled nuclei*

Autoradiography was carried out by incubating quiescent SMCs with DMEM containing 0.2% calf serum and 5 $\mu$Ci/ml $^3$H-thymidine in the presence and absence of exogenous growth factors for 24 h. At this time, plates were washed extensively with PBS, fixed with methanol (10 min, 4°C), and dried. The dishes were then coated with Ilford K5 photographic emulsion, incubated for 5 d, and developed with Kodak D-19 developer.

### *RNA isolation*

RNA was isolated according to the method of Chomczynski and Sacchi (1987). Cell monolayers were solubilized in a minimal volume (0.5–1 ml) of solution containing 4 M guanidinium thiocyanate (Fluka), 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.092 M 2-mercaptoethanol. The solubilized material was incubated overnight at 37°C. Extraction with phenol and chloroform was then performed, and the RNA-containing aqueous phase was precipitated in ethanol.

### *Capillary transfer of RNA to GeneScreen (Northern blot)*

Fractionation of RNA on an agarose gel was carried out by modification of a described method (Lehrach *et al.*, 1977). Briefly, RNA samples were separated on 1% agarose gel containing 1.1M formaldehyde. After electrophoresis, the gel was transferred to GeneScreen membrane in 20× SSPE (174 g NaCl, 27.6 g $NaH_2PO_4$ $H_2O$, and 7.4 g EDTA per 1 l, pH 7.4) and baked in a vaccum oven at 80°C for 2 h. Detection of target RNA species was carried out after hybridization with randomly labeled $^{32}$P-*c-fos* DNA probe (American Type Culture Collection, isolated from human lymphoblast cell line CCRF-CM) with the use of $^{32}$P-dCTP (Pharmacia; Upssala, Sweden) at $\sim$0.5–1 $\times$ $10^7$ cpm/filter. Hybridization was carried out with 33% formamide at 42°C for cross-species hybridization. Specifically hybridized RNA was detected on exposure to X-ray film.

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