Monoclonal Antibody to the Thrombin Receptor Stimulates DNA Synthesis in Combination with Gamma-Thrombin or Phorbol Myristate Acetate

Gloria H. Frost, William C. Thompson, and Darrell H. Carney

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550

Abstract. Studies with various thrombin derivatives have shown that initiation of cell proliferation by thrombin requires two separate types of signals: one, generated by high affinity interaction of thrombin or DIP-thrombin (alpha-thrombin inactivated at ser 205 of the B chain by diisopropylphosphofluoridate) with receptors and the other, by thrombin's enzymic activity. To further study the role of high affinity thrombin receptors in initiation, we immunized mice with whole human fibroblasts and selected antibodies that blocked the binding of ^{125}I-thrombin to high affinity receptors on hamster fibroblasts. One of these antibodies, TR-9, inhibits from 80 to 100% of ^{125}I-thrombin binding, exhibits an immunofluorescent pattern indistinguishable from that of thrombin bound to receptors on these cells, and selectively binds solubilized thrombin receptors. By itself, TR-9 did not initiate DNA synthesis nor did it block thrombin initiation, but TR-9 addition to cells in the presence of alpha-thrombin, gamma-thrombin (0.5 μg/ml), or PMA stimulated thymidine incorporation up to threefold over controls. In all cases, maximal stimulation was observed at concentrations of TR-9, ranging from 1 to 4 nM corresponding to concentrations required to inhibit from 30 to 100% of ^{125}I-thrombin binding. These results demonstrate that the binding of the monoclonal antibody to the alpha-thrombin receptor can mimic the effects of thrombin's high affinity interaction with this receptor in stimulating cell proliferation.

HIGHLY purified thrombin initiates proliferation of fibroblastic cells in serum-free medium without addition of other growth factors (14, 21, 46–48, 60), initiates proliferation of mammalian lens epithelial cells (50), and stimulates DNA synthesis in spleen cells (22). Thrombin also acts synergistically with other growth factors to stimulate proliferation of hamster fibroblasts (23) and human endothelial cells (33, 59). More recently, thrombin has been shown to act as a chemoattractant to stimulate cell migration (4–6). Therefore, thrombin may play a significant role in several aspects of wound healing when presented to cells after tissue injury.

Early studies demonstrated that the action of thrombin at the cell surface is sufficient to initiate cell proliferation (13, 15) and that this thrombin interaction appears to involve specific high affinity receptors (14). A number of studies have been carried out to characterize these receptors and the interaction of thrombin with fibroblasts. Photoaffinity studies with fibroblasts have shown active thrombin and proteolytically inhibited thrombin cross-linking to molecules of approximate 50,000 (17) and 150,000 (43, 56) M_r. HPLC size exclusion and HPLC immunoaffinity purification of the thrombin receptor also indicate that fractions retaining thrombin receptor binding activity contain molecules corresponding to 150,000, 105,000, and 55,000 M_r (Frost, G. H., and D. H. Carney, manuscript in preparation). Thus, it is likely that the intact receptor may have multiple subunits or a proteolytically labile region similar to the insulin receptor (25).

Studies with indirect immunofluorescence microscopy, immunocytochemistry, and ultrastructural autoradiography have shown that these receptors, unlike receptors for epidermal growth-factor (EGF)[1] and insulin, are clustered on the surface of cells before thrombin binding (7, 11, 12). These receptors and the clusters appear to be excluded from coated pits (12). Furthermore, ^{125}I-thrombin bound to these receptors is not rapidly internalized and degraded (7). Instead, degradation of thrombin occurs after thrombin complexes to protease-nexin (PN) and is internalized via PN receptors which are not involved in thrombin mitogenesis (2, 3, 38). Thus, thrombin receptors, interaction of thrombin with these receptors, and the transmembrane signals generated by this interaction may be somewhat different from that observed with other growth factors.

Consistent with thrombin's enzymic role as a serine protease, thrombin mitogenesis appears to require both high affinity receptor occupancy and enzymatic cleavage of the

---

1. *Abbreviations used in this paper*: DIP-thrombin, alpha-thrombin inactivated at ser 205 of the B chain by diisopropylphofluoridate; DV, Dulbecco-Vogt modified Eagle's medium; EGF, epidermal growth factor; PN, protease-nexin.

receptor or some other molecule on the cell surface. Proteolytically inactive DIP-thrombin (alpha-thrombin inactivated at ser 205 of the B chain by diisopropylphosphofluoridate) binds to thrombin receptors on mouse embryo and hamster cells as well as native alpha-thrombin, but by itself does not initiate cell proliferation (29). Likewise, gamma-thrombin, which retains enzymic activity but not the potential for high affinity occupancy, does not by itself initiate cell proliferation. In the presence of receptor-saturating concentrations of DIP-thrombin, however, gamma-thrombin initiates cell proliferation to levels approaching maximal stimulation by alpha-thrombin (20). Thus, both receptor occupancy and enzymic cleavage and the signals generated by each appear to be required to stimulate cell proliferation (18, 20).

The use of intact alpha-thrombin and these thrombin derivatives has facilitated analysis of the role of receptor occupancy and proteolysis in relation to transmembrane signal events that may be involved in mitogenesis. In this system, phosphoinositide turnover (19), Ca^{++} mobilization (Carney, D., et al., manuscript in preparation), and stimulation of Na^+/H^+ antiport (52, 53) all appear to be stimulated by alpha- or gamma-thrombin but not by DIP-thrombin (18). Since PMA can also stimulate Na^+/H^+ antiport (9) we postulated that the activation of protein kinase C may be important in mediating thrombin's enzymic effects on these cells. We recently showed that the addition of PMA to cells in combination with DIP-thrombin stimulates DNA synthesis and cell proliferation as well as the combination of DIP- and gamma-thrombin (31). Thus, it appears that PMA activation of protein kinase C can mimic the enzymic effects of gamma-thrombin in completing a mitogenic signal. In contrast, receptor occupancy by DIP- or alpha-thrombin stimulates a transient increase in cAMP, but cAMP derivatives cannot stimulate cell proliferation in combination with gamma-thrombin or PMA (32). This suggests that high affinity interaction of thrombin with its receptor may generate conformational changes in the receptor and/or additional signals which are required for thrombin mitogenesis.

To more fully define the thrombin receptor responsible for these signals and to understand the nature of the signals generated by high affinity receptor interaction, we have prepared monoclonal antibodies to the thrombin receptor. We now show that these antibodies initiate DNA synthesis in the presence of esterolytically active thrombin or PMA.

Materials and Methods

Materials

Human alpha-thrombin of high purity ($\sim$3,000 NIH U/μg) was supplied by Dr. J. W. Fenton II (New York Department of Health, Albany, NY). Unless otherwise stated, all common chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Plastic tissue culture dishes were obtained from Corning Glass Works, Corning Science Products, Corning, NY. $Na^{125}I$ (100 Ci/mmol, 17 Ci/μg free iodine) was purchased from Amersham Corp., Arlington Heights, IL.

Cell Cultures

Human fibroblasts used to immunize mice were prepared from cells migrating out of minced human foreskin tissues, and primary cultures of fibroblasts were prepared from 9–13-d-old embryos of Swiss outbred mice, as previously described (16). NIL cells are an established strain of Syrian hamster fibroblasts provided by Frank Solomon (Massachusetts Institute of Technology, Cambridge, MA). Faza rat hepatoma cells were given to us by

Gerald Fuller (University of Alabama, Birmingham, AL). These cells were maintained and grown in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium (DV/F₁₂; Gibco, Grand Island, NY) supplemented with 10% calf serum or, in the case of Faza cells, FBS (Hyclone Laboratories, Logan, UT), penicillin (100 U/ml), and streptomycin (100 μg/ml), in a humidified atmosphere of 5% CO_2 in air at 37°C.

Nonproliferating quiescent cultures of these cells were prepared by subculturing stock cells from 100-mm dishes with an EDTA/trypsin solution (PBS containing 0.2% EDTA and 0.05% trypsin) and plating them onto 24-well culture plates or glass coverslips in DV/F₁₂ medium supplemented with 10% calf serum or FBS at 6.5×10^4 cells/cm². After cell attachment, the medium was removed and the cells were rinsed with serum-free DV/F₁₂ medium and incubated in this serum-free medium for 48 h to bring the cells to a quiescent G_1/G_0 arrested state (24).

Immunization Procedure

BALB/c mice were injected intraperitoneally with 10^7 human foreskin fibroblasts two times at 4-wk intervals. 2 mo after the last injection, the mice received a booster injection and were killed 3 d later for the cell fusion.

Hybridization and Selection of Hybridomas

Spleen cells from the immunized mice were fused with P₃ myeloma cells (36) at a 5:1 ratio following the procedure of Galfre et al. (28) and using 41% (vol/vol) polyethylene glycol 1500 as the fusing agent. Hybrid cells growing in hypoxanthine/aminopterin/thymidine selective medium were initially screened for their ability to inhibit ^{125}I-thrombin binding and for their pattern of binding to the surface of formaldehyde-fixed mouse embryo cells. Cells from selected clones were subcloned and initially cultured in DV/F₁₂ medium supplemented with 17% horse serum (Irvine Scientific, Santa Anna, CA). To reduce the complication of serum factors that bind to thrombin receptors (8), promising clones were cultured in 17% Nu Serum (Collaborative Research, Inc., Waltham, MA) or injected into isogenic BALB/c mice for ascites production and antibody purification.

Purification of Antibody from Ascites Fluid

TR-9 IgM monoclonal antibodies were purified using a modification of the procedure described by Bouvet et al. (10). Briefly, ascites fluid was centrifuged at 10,000 g for 30 min to remove particulate material. The precipitate from a 30–50% ammonium sulfate precipitation was dissolved and applied to a Sephacryl S-200 column and eluted in PBS. Fractions containing IgM located in the void volume by a solid phase radioimmunoassay using ^{125}I-goat anti-mouse IgM (Cappel Scientific Div., CooperBiomedical, Inc., Malvern, PA) were pooled, concentrated, dialyzed, and applied to a second Sephacryl S-200 column equilibrated in 0.005 M phosphate buffer, pH 8.0, and eluted with 0.05 M phosphate buffer, pH 8.0, containing 1.7 M NaCl. IgM antibodies precipitate in this low salt column and elute after resolubilization with increasing salt concentration. Thus the majority of material elutes in the void volume while the IgM fraction elutes as a second peak. The purity of the IgM fraction at the last stage of the purification was >90–95% based on analysis of SDS-polyacrylamide gels (37) and reverse phase HPLC.

Immunofluorescence Microscopy

Secondary cultures of fibroblasts were plated on glass coverslips (1×10^4 cells/cm²) and prepared for immunofluorescence staining as described by Carney (11). The fibroblasts were fixed in 3% formaldehyde and incubated with either thrombin (60 ng/ml) and affinity-purified rabbit antithrombin or mouse ascites diluted in 15 mM Hepes buffered DV medium (pH 7.4) containing 0.5% BSA. The cells were then stained with anti-mouse or anti-rabbit rhodamine-labeled antibody (Cappel Scientific Div., CooperBiomedical, Inc.).

Iodination of Thrombin and TR-9

Purified thrombin was iodinated by the lactoperoxidase method as described previously (12, 14). Purified TR-9 IgM antibodies (150 μg) were iodinated in the presence of 20 μg Iodo-gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Pierce Chemical Co., Rockford, IL). The labeled antibodies were separated from unreacted iodide by gel filtration in a Sephadex G-25 M prepacked column (Pharmacia, Uppsala, Sweden) that had been washed and equilibrated with PBS containing 5% BSA (wt/vol) and eluted with PBS. The specific activity of ^{125}I-TR-9 was 1×10^6 cpm per μg protein.

Binding Assays

[125]I-Thrombin binding to fibroblasts in the absence or presence of monoclonal antibodies was measured with nonproliferating cultures of NIL hamster cells in 24- or 48-well plates at a density of ~6 × 10⁴ cells/cm² as previously described (14). The medium on the cells was changed to binding medium (serum-free DV medium containing 0.5% BSA buffered with 15 mM Hepes at pH 7.0). The cells were equilibrated at 23°C for 30 min and the medium was changed to binding medium containing various concentrations of antibody. The cells were preincubated with antibody for 2 h at 23°C, after which ^{125}I-thrombin was added (final concentration, 30 ng/ml) and the cells incubated for another 2 h at 23°C. The assay was terminated by quickly rinsing the cells four times with cold PBS. The cells were dissolved in 1 ml of 0.5 N NaOH and the total radioactivity was determined. Nonspecific binding was measured as the radioactivity bound to cultures after incubation in binding medium containing excess unlabeled thrombin (3 μg/ml). Specific binding was determined by subtracting nonspecific binding from total radioactivity bound to cultures. Total and nonspecific binding were each measured on duplicate cultures. Specific binding normally represents ~80% of the total ^{125}I-thrombin bound in control wells. Under these binding conditions, SDS gel electrophoresis shows little, if any, ^{125}I-thrombin-PN complex formation. Thus, under these conditions, specific ^{125}I-thrombin binding primarily represents thrombin interaction with its high affinity receptors.

^{125}I-TR-9 binding to fibroblasts in the presence or absence of alpha-thrombin was measured in the same manner as described for ^{125}I-thrombin.

Immunoaffinity Chromatography

Purified TR-9 monoclonal antibody (2 mg) was covalently linked to cyanogen bromide–activated Sepharose CL 4B (10 ml) (Pharmacia Inc., Piscataway, NJ). The TR-9-Sepharose was washed, blocked with 1 M glycine, and then incubated overnight with a mixture of membrane proteins that had been extracted from fibroblasts with PBS containing 10 mM (3[3 cholamidopropyl] dimethylammonio-l-propanesulfonate), 25% glycerol, 1 mM dithiothreitol, 1 mM EDTA and various protease inhibitors, i.e., antipain (20 μg/ml), aprotinin (0.1 trypsin inhibitor U/ml), leupeptin (20 μg/ml), pepstatin (20 μg/ml) and phenylmethylsulfonyl fluoride (1 mM). After incubation, the affinity column was washed and bound protein was eluted with 0.1 M citrate/500 mM NaCl buffer, pH 2.0. The eluted protein fractions were then adsorbed to plastic in 24-well clusters overnight at 37°C and tested for their ability to specifically bind ^{125}I-thrombin as described above.

SDS-PAGE Analysis

Cell surface proteins that bound to TR-9-Sepharose were analyzed by electrophoresis in polyacrylamide slab gels (5% acrylamide) with 0.1% SDS in 0.1 M Tris-acetate buffer, pH 8.2, in both gel and electrode buffer (58). After electrophoresis, the gels were stained with 0.05% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA) dissolved in 50% 2-propanol and destained with 10% acetic acid/10% 2-propanol.

Measurement of DNA Synthesis

The effects of TR-9 on DNA synthesis, either alone or in combination with gamma-thrombin, were determined by measuring the incorporation of methyl-[³H]thymidine (TdR; ICN Pharmaceuticals, Irvine, CA) during a 30-min incubation for 23.5–24 h after addition of TR-9 and/or alpha-thrombin, gamma-thrombin, or PMA. After incubation, the cells were extracted and rinsed with cold 10% TCA. The acid-precipitable material was dissolved overnight in 0.5 ml KOH (0.5N) at 23°C. 0.25 ml HCl (1N) was added and the solution was counted in 10 ml of scintillation fluid (Beckman ReadySolv-HPb; Beckman Instruments, Houston, TX).

Results

Selection and Characterization of Monoclonal Antireceptor Antibodies

To better characterize the high affinity thrombin receptor and define its role in mediating the mitogenic effect of thrombin, we prepared monoclonal antibodies to this receptor. Since a purified receptor was unavailable, mice were immunized by injection of whole human foreskin fibroblasts. After the fusion, supernatants from ~200 hybridoma clones were screened for inhibition of ^{125}I-thrombin binding to mouse embryo or NIL hamster cells. Approximately 30 clones exhibiting inhibition over control supernatants were expanded and further analyzed by comparing the immunofluorescent staining pattern of the antibodies to that of thrombin on fixed cells. As shown in Fig. 1, thrombin binding to both HF cells originally injected into mice for immunization (Fig. 1 A),
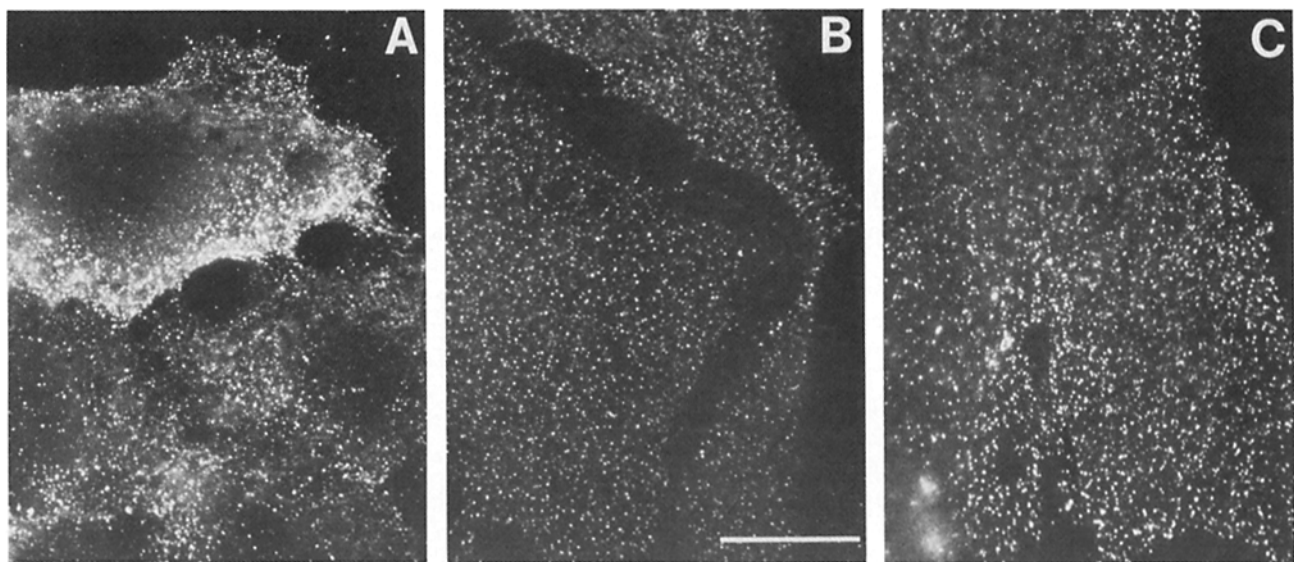
Figure 1. Immunofluorescent staining of thrombin and TR-9 monoclonal antibodies on human and NIL hamster cells. Coverslip cultures of human fibroblasts (*A*) or NIL hamster cells (*B* and *C*) were fixed in 3% formaldehyde and incubated with thrombin (*A* and *B*) followed by polyclonal rabbit anti–thrombin (*B*) or TR-9 monoclonal antibody (*C*). Cells were stained with anti–mouse or anti–rabbit rhodamine-labeled second antibody and photographed as described in Materials and Methods. Bar, 20 μm.
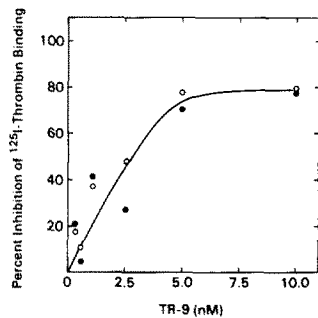
Figure 2. Inhibition of ^{125}I-thrombin binding to hamster fibroblasts by purified TR-9 IgM antibodies. The binding of 30 ng/ml of ^{125}I-thrombin to live (o) and 3% formaldehyde-fixed (●) hamster fibroblasts was inhibited by increasing concentrations of TR-9 IgM.

and NIL hamster fibroblasts (Fig. 1 *B*) can be visualized with antithrombin polyclonal antibodies as a distinct pattern of dots, each ∼400 nm in diameter. Previous studies have estimated that each of these clusters contains from 200 to 1,000 thrombin receptors (11, 12). It should be noted that this staining pattern is quite different from that of EGF or insulin which appear to initially bind to diffuse receptors (35, 41, 44). Furthermore, other receptors that cluster before ligand binding of molecules such as low density lipoprotein or asialoorosomucoid have clusters in coated pits which appear to be larger and generally concentrated in these cells near the nucleus (1, 12, 30, 45, 57).

Of the 30 clones analyzed for their immunofluorescent staining pattern, 19 had diffuse or negative staining patterns suggesting that their inhibition of ^{125}I-thrombin binding may have been due to nonspecific blocking of receptors or interaction with thrombin before binding. 11 clones had dotlike patterns of various intensities. Of these, four appeared to have large perinuclear dots characteristic of binding to receptors in coated pits and seven exhibited binding patterns similar to the binding of thrombin to its receptor. One of these, an IgM producer designated TR-9, exhibited strong growth, high binding competition, and consistent dotlike staining pattern indistinguishable from that of thrombin bound to its receptors (Fig. 1 *C*).

The diffuse binding or binding to large perinuclear dots observed with some clones that blocked thrombin binding may have represented binding to protease nexin or to PN receptors. The dot pattern of TR-9 binding, however, appears to be quite characteristic of high affinity thrombin receptor binding. Previous studies have shown that high concentrations of heparin that block thrombin–PN complex binding to PN receptors (2) have no effect on thrombin binding to its high affinity receptors and does not affect thrombin binding to the clustered receptors visualized by immunofluorescence microscopy (11). Thus, the dot pattern observed with TR-9 (Fig. 1 *C*) is characteristic of thrombin binding to high affinity receptors and not to PN or to PN receptors.

Further evidence for the specificity of TR-9 was obtained after subcloning, production of ascites fluid, and purification of TR-9. As shown in Fig. 2, pretreatment of NIL cells (live or prefixed with 3% formaldehyde in parallel to the immunofluorescence binding) with purified TR-9 inhibits ^{125}I-thrombin binding up to 80% with half-maximal inhibition occurring at a concentration of ∼2 nM. Under these binding conditions, where cells are rinsed free of conditioned medium containing protease nexin, previous studies have failed to detect ^{125}I-thrombin–PN complexes contributing to the specific binding. In addition, in several experiments we

have observed up to 100% inhibition of the specific binding of thrombin to NIL cells by TR-9, indicating that at these thrombin concentrations all binding is to a single specific receptor recognized by TR-9.

To further establish that TR-9 recognizes the thrombin receptor, we iodinated purified TR-9 and compared its binding to different cells with that of ^{125}I-thrombin. Table I summarizes the estimations of total binding sites from Scatchard plots for three different cell types. As shown, there appears to be a positive correlation between immunoblotting to putative thrombin receptor bands on SDS gels, number of binding sites for thrombin, and the number of binding sites for TR-9. Both NIL hamster fibroblasts and B11-C's, an established line of mouse embryo cells initially selected for high numbers of thrombin binding sites (54), have ∼4–5 × 10⁵ binding sites for both thrombin and purified TR-9. In contrast, Faza cells, an established rat hepatoma cell line (40), show negative immunoblotting of putative thrombin receptors and have less than a few thousand thrombin or TR-9 binding sites. Thus, direct binding of TR-9 correlates with binding of ^{125}I-thrombin. Furthermore, in both NIL and B11-C cells, unlabeled thrombin partially inhibits binding of ^{125}I-labeled TR-9 (data not presented). Thus, it seems likely that TR-9 recognizes an epitope on the thrombin receptor near, but perhaps not identical to, the domain responsible for high affinity thrombin binding.

Table I. Comparison of ^{125}I-Thrombin and ^{125}I-TR-9 Binding to Thrombin Receptor-positive and -negative Cells

Cell type	TR-9 immunoblotting*	Thrombin-binding sites‡	TR-9-binding sites‡
NIL cells	+	4.0 × 10⁵	4.1 × 10⁵
B11-C cells	+	5.0 × 10⁵	4.6 × 10⁵
Faza cells	−	Not detectable	2.3 × 10³

* Nonproliferating cultures of NIL hamster cells, B11-C cells, or Faza rat liver hepatoma cells in 100-mm tissue culture dishes (4 × 10⁶ cells) were solubilized in SDS-PAGE sample buffer and subjected to electrophoresis in 5% polyacrylamide gels as described in Materials and Methods. The separated proteins were then transferred from the gel into nitrocellulose according to the method of Towbin et al. (55). After transfer, TR-9-binding sites were visualized with the Immun-blot (GAR-HRP) assay kit (Bio-Rad Laboratories).
‡ These numbers were obtained from the Scatchard type analysis of the binding of various concentrations of ^{125}I-thrombin (15–500 ng/ml) or ^{125}I-TR-9 (0.25–8 nM) to nonproliferating cultures of NIL hamster cells, B11-C cells, or Faza rat liver hepatoma cells in 24-well plates at a density of ∼6 × 10⁴ cells/cm² as described previously (14).

Table II. Binding of ^{125}I-Thrombin to TR-9-purified Fractions Adsorbed to Plastic

Preparation	Specific ^{125}I-thrombin binding*
	cpm/μg protein
NIL cell total extract	33.0 ± 0.2
TR-9 affinity column	
Nonadsorbed material	46.2 ± 8
Adsorbed material (removed with citrate/NaCl)	439.3 ± 252

* Radioactivity is expressed as cpm per μg protein initially added to each well. Each value represents the mean of triplicate determinations plus or minus the standard deviation.
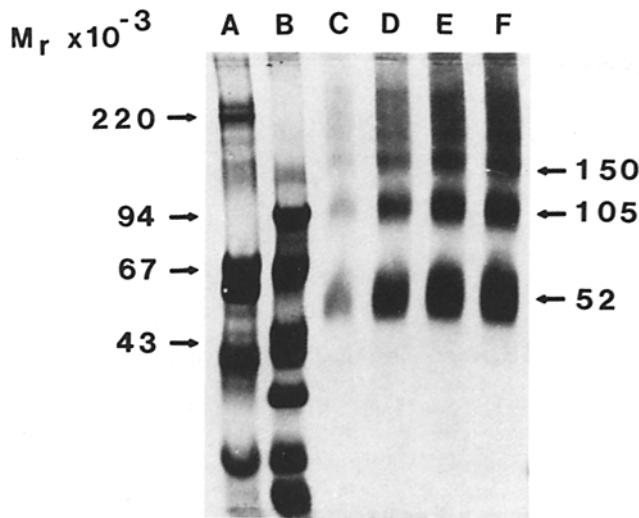
Figure 3. SDS-PAGE analysis of proteins bound to TR-9 Sepharose column. Eluted proteins from the TR-9-Sepharose were subjected to SDS-PAGE as described in Materials and Methods. Before electrophoresis, the samples were boiled in Tris-acetate buffer for 5 min in the presence of 0.1% β-mercaptoethanol. Molecular mass standards were applied to lanes A and B. Lanes C-F contain increasing concentrations of pooled material eluted from the column.

If TR-9 binds an epitope on thrombin receptors on cell surfaces, one might expect this antibody to also selectively bind solubilized receptors. To test this, we coupled purified TR-9 to Sepharose, and incubated the TR-9-Sepharose with 3(3 cholamidopropyl)dimethylammonio-1-propanesulfonate extracts of NIL cells, and eluted any bound material with low pH and high salt buffer or with potassium isothiocyanate. As shown in Table II, material that selectively adsorbed to this column retained ^{125}I-thrombin-binding activity with approximately a 10-fold higher specific activity than nonadsorbed material. SDS gels of this material indicate the presence of three major proteins that migrate at approximately 52,000,
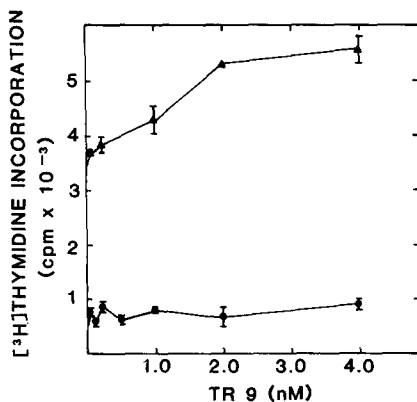

Figure 4. Effect of purified TR-9 monoclonal IgM on stimulation of [^{3}H]thymidine incorporation by alpha-thrombin. Quiescent serum-free cultures of NIL hamster fibroblasts were incubated at 37°C for 23 h with increasing concentrations of purified TR-9 (the IgM fraction isolated from TR-9 ascites) in the presence (▲) or absence (●) of thrombin (125 ng/ml) before a 1-h pulse with [methyl-^{3}H]thymidine and determination of acid-precipitable radioactivity.
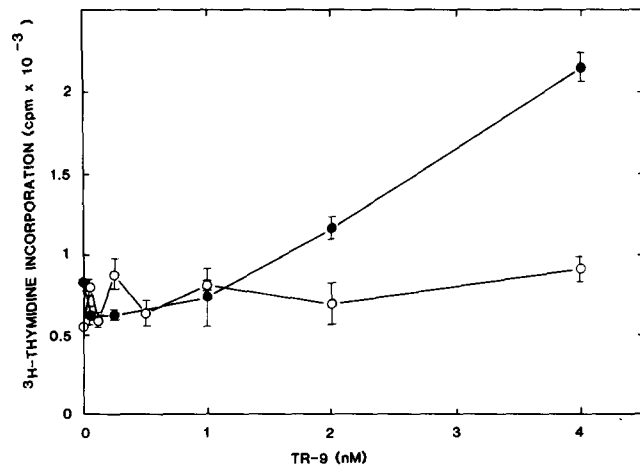

Figure 5. Stimulation of [^{3}H]thymidine incorporation by purified TR-9 monoclonal IgM in combination with gamma-thrombin. Quiescent cultures of hamster fibroblasts were incubated for 23 h with the indicated concentrations of purified TR-9 either with (●) or without (○) gamma-thrombin (0.5 μg/ml) before a 1-h pulse with [methyl-^{3}H]thymidine. The amount of radioactivity in the acid-precipitable material was determined.

105,000, and 150,000 M_r (Fig. 3). Since previous studies have demonstrated ^{125}I-thrombin cross-linking to molecules of 50,000 and 150,000 M_r (17, 43, 56), it is possible that both 150,000- and 52,000-M_r peptides have thrombin-binding domains that might also be recognized by TR-9. Indeed, immunoblots of whole cell extracts and material eluting from TR-9-Sepharose have shown TR-9 binding to both the 150,000-M_r band and more prominently to the 52,000-M_r band. Most important for our present characterization, it appears that TR-9 does not bind other molecules that are known to bind thrombin such as PN (46,000 M_r) (2) or thrombomodulin (96,000 M_r) (26).

Mitogenic Potential of TR-9 Monoclonal Antibody

Since TR-9 recognizes thrombin receptors and could effectively compete for up to 100% of thrombin binding, we determined if this antibody could also block the biological effects of thrombin. As shown in Fig. 4, the addition of TR-9-IgM by itself does not initiate DNA synthesis in cultures of fibroblastic cells at concentrations up to 4 nM. Interestingly, if TR-9 is added to cells with concentrations of thrombin previously shown to be half-maximal for initiating DNA synthesis under these conditions (20), there is no inhibition of thrombin mitogenic activity (Fig. 4). Rather, TR-9-IgM appears to enhance thrombin stimulation of thymidine incorporation from a 7-fold stimulation (by thrombin alone) to an 11-fold stimulation with thrombin in the presence of 4 nM TR-9. Thus, unlike some antibodies that produce inhibitory effects by limiting the interaction of growth factors with their receptors (27, 42), TR-9 appears to enhance this initiation. This suggested that TR-9 interaction with thrombin receptors might be generating receptor occupancy–related signals, which are required for thrombin mitogenesis.

To determine if TR-9 binding to the receptors was generating receptor occupancy–dependent signals, TR-9-IgM was added to cells together with gamma-thrombin, which retains enzymic activity, but does not bind to cells with high affinity. As shown in Fig. 5, neither TR-9 nor gamma-thrombin
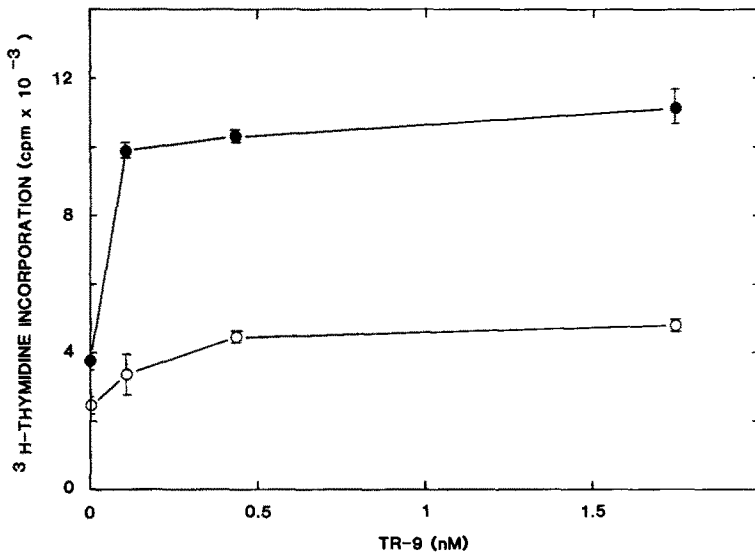
Figure 6. Stimulation of [³H]thymidine incorporation by purified TR-9 monoclonal IgM in combination with PMA. Quiescent cultures of hamster fibroblasts were incubated for 23 h with indicated concentrations of purified TR-9 either with (●) or without (○) PMA (25 ng/ml) before a 1-h pulse with [methyl-³H]thymidine. The amount of radioactivity in the acid-precipitable material was determined.

stimulated thymidine incorporation by themselves, but the combination causes a threefold stimulation of thymidine incorporation which is nearly equivalent to that seen with combinations of gamma-thrombin and DIP-thrombin (20).

The above results indicate that the TR-9 antibody behaves like DIP-thrombin in that it fulfills the requirement for high affinity receptor interaction in the generation of mitogenic signals. TR-9 interaction with the receptor, however, could alter the receptor conformation allowing gamma-thrombin to generate both enzymic and receptor occupancy–related signals. Recent studies in our laboratory have shown that PMA or dioctanoylglycerol, which activate protein kinase C, can stimulate DNA synthesis in the presence of receptor-saturating concentrations of DIP-thrombin (31). Thus, activation of protein kinase C appears to mimic mitogenic signals stimulated by gamma-thrombin. If TR-9 were truly generating mitogenic signals itself, we predicted that combinations of TR-9 and PMA should be equally as effective as TR-9 and gamma-thrombin in stimulating DNA synthesis.

As shown in Fig. 6, 25 ng/ml of PMA by itself has little, if any, effect on DNA synthesis, but when added to cultures together with TR-9-IgM there is a synergistic initiation comparable to that achieved with intact alpha-thrombin or to the combination of DIP- and gamma-thrombin. In this case, there were no active thrombin molecules present to provide mitogenic signals. Thus, the TR-9 monoclonal antibody interaction with thrombin receptors alone generates signals that are necessary, and in the presence of PMA, sufficient to commit cells to enter a proliferative cycle.

Discussion

To better characterize the high affinity receptor for thrombin and its involvement in initiation of cell proliferation we prepared and selected a monoclonal antibody to this receptor and tested its effect on thrombin mitogenesis. The selected antibody, designated TR-9, inhibits from 80 to 100% of the specific ¹²⁵I-thrombin binding to fibroblasts, has an immunofluorescent-staining pattern indistinguishable from that of thrombin binding, binds to the same number of sites as does thrombin, and selectively affinity purifies a putative recep-

tor complex which retains the ability to bind ¹²⁵I-thrombin. Thus, this antibody clearly recognizes the high affinity thrombin receptor. By itself, TR-9 did not stimulate DNA synthesis nor block stimulation by alpha-thrombin. Instead, it enhanced stimulation by half-maximal concentrations of alpha-thrombin. Moreover, in combination with gamma-thrombin or PMA, TR-9 stimulated DNA synthesis to a degree nearly equivalent to that achieved by active alpha-thrombin.

These results with TR-9 monoclonal antibody are consistent with the "double signal hypothesis" model previously proposed to explain the mechanism of thrombin mitogenesis (18). Previous studies from our laboratory have shown that cell proliferation can be initiated by the addition of DIP-thrombin, which binds to thrombin receptors with high affinity, and gamma-thrombin, which does not interact with high affinity, but retains its full esterase activity (20). In the present studies, addition of TR-9-IgM and proteolytically active gamma-thrombin or PMA, which appears to mimic the signal generated by proteolysis (31), stimulated DNA synthesis to levels comparable to those achieved with DIP-thrombin and gamma-thrombin. This indicates that these combinations provide all signals necessary to stimulate mitogenesis. Thus, TR-9 binding is capable of generating the high affinity occupancy dependent mitogenic signals.

The similarity in action of DIP-thrombin and TR-9-IgM which, molecularly, are similar only in their ability to bind the thrombin receptor, further indicates the importance of the thrombin receptor in the initiation of cell proliferation. Other investigators suggest that occupancy of the thrombin receptor is not necessary for mitogenic stimulation (39). They report a subclone of Chinese hamster fibroblasts (11C-9) that are highly responsive to thrombin but exhibit little high affinity ¹²⁵I-thrombin binding. Our preliminary results show that these cells have large numbers of low affinity thrombin receptors that also bind the TR-9 monoclonal antibody. It therefore seems likely that these receptors may be altered in some way to cause a lower affinity of ¹²⁵I-thrombin binding. This same alteration (perhaps a conformational change) may result in constitutive activation of certain required signals normally generated by high affinity receptor

2556

occupancy. Additional studies with these important cells may help solve this apparent conflict.

Other anti–growth factor receptor monoclonal and polyclonal antibodies have been generated and studied to determine their ability to mediate growth effects of the corresponding growth factors. In some cases, antireceptor antibodies inhibit both receptor binding and stimulation of DNA synthesis by these growth factors (27, 32, 49). In contrast anti–EGF receptor antibody 2G2IgM inhibits the binding of ^{125}I-EGF to its receptors but induces "EGF-like" responses including morphological changes, stimulation of the EGF receptor–specific protein kinase, and mitogenesis (51). In these and other studies, most antibodies capable of generating mitogenic signals appear to be multivalent IgM antibodies, whereas, most IgGs have been inactive. It is possible that the decavalent IgM antibodies have lower dissociation constants and/or are better able to cross-link receptors to generate necessary changes in the density or conformation of the receptors.

Interestingly, TR-9 monoclonal antibodies used in this study also belong to the IgM class. These antibodies may thus cross-link or perturb thrombin receptors to activate mitogenic signals. We have postulated that binding of thrombin, DIP-thrombin or TR-9 causes a conformational change in the receptor that gives rise to other events, which include a transient increase in intracellular cAMP (18, 32). Indeed, our preliminary results indicate that TR-9-IgM also is capable of causing a transient increase in intracellular cAMP. Addition of cAMP derivatives to these cells, however, cannot substitute for DIP-thrombin or TR-9. Thus, the interaction of these molecules with the receptor must also generate additional signals.

Thrombin stimulates a number of additional events that play a role in thrombin mitogenesis. Thrombin stimulates turnover of phosphoinositides, release of inositol phosphates (19), and activates Na$^+$/H$^+$ exchange (52, 53). These signal events, however, appear to be generated by active thrombin proteolysis rather than receptor occupancy since they can be stimulated by gamma-thrombin but not by DIP-thrombin (18, 49, 52). Since phosphoinositide turnover releases diacylglycerol which can activate protein kinase C and protein kinase C activation by PMA has been implicated in Na$^+$/H$^+$ antiport activation (9), we postulated that activation of protein kinase C may play a crucial role in thrombin mitogenesis. We have now shown that PMA can substitute for enzymatically active alpha- or gamma-thrombin in stimulating cell proliferation when thrombin receptors are occupied by TR-9-IgM or by DIP-thrombin (20). In the present experiments, PMA treatment also appears to reduce the concentration of TR-9 necessary to complete mitogenic signals relative to that required when cells are incubated with TR-9 plus gamma-thrombin. This may indicate that PMA is a much better activator of protein kinase C or that the PMA-induced protein kinase C activation triggers other events that either alter the affinity of the receptor for antibody interaction or the efficiency of receptor-mediated signal transduction.

Although the mechanism by which receptor occupancy or proteolytic cleavage stimulates mitogenic signal events is not yet clear, the discovery that monoclonal antibodies to thrombin receptors by themselves can generate mitogenic signals suggests that some of these signals can be generated simply by perturbing the conformation of the receptor. Experiments are presently underway to use these monoclonal antibodies to purify and further characterize the thrombin receptor to better understand this process.

References

1. Anderson, R. G. W., E. Vasille, R. J. Mellow, M. S. Brown, and J. Goldstein. 1978. Immunocytochemical visualization of coated pits and vesicles in human fibroblasts: relation to low density lipoprotein receptor distribution. *Cell.* 15:919–933.
2. Baker, J. B., D. A. Low, D. L. Eaton, and D. D. Cunningham. 1982. Thrombin-mediated mitogenesis: the role of secreted protease-nexin. *J. Cell. Physiol.* 112:291–297.
3. Baker, J. B., D. A. Low, R. L. Simmer, and D. D. Cunningham. 1980. Protease-nexin: a cellular component that links thrombin and plasminogen activator and mediates their binding to cells. *Cell.* 21:37–45.
4. Bar-Shavit, R., D. H. Bing, A. J. Kahn, and G. D. Wilner. 1985. Thrombin-mediated chemotaxis: relationship of ligand structure to biological activity. *In* Membrane Receptors and Cellular Regulation. C. F. Fox, editor. Alan R. Liss, Inc., New York. 329–338.
5. Bar-Shavit, R., A. Kahn, J. W. Fenton II, and G. D. Wilner. 1983. Chemotactic response of monocytes to thrombin. *J. Cell Biol.* 96:282–285.
6. Bar-Shavit, R., A. Kahn, J. W. Fenton II, and G. D. Wilner. 1983. Receptor-mediated chemotactic response of a macrophage cell line (J774) to thrombin. *Lab. Invest.* 49:702–707.
7. Bergmann, J. S., and D. H. Carney. 1982. Receptor bound thrombin is not internalized through coated pits in mouse embryo cells. *J. Cell. Biochem.* 20:247–258.
8. Bergmann, J. S., I. Thomas, and D. H. Carney. 1983. Partial purification of a fibroblast growth factor from calf and horse serum which utilizes the thrombin receptor system. *J. Cell Biol.* 97:394a. (Abstr.)
9. Besterman, J. A., and P. Cautrecasas. 1984. Phorbol esters rapidly stimulate amiloride-sensitive Na$^+$/H$^+$ exchange in human leukemic cell line. *J. Cell Biol.* 99:340–343.
10. Bouvet, S. P., R. Pires, and J. Pillot. 1984. A modified gel filtration technique producing an unusual exclusion volume of IgM: a simple way of preparing monoclonal IgM. *J. Immunol. Methods.* 66:299–305.
11. Carney, D. H. 1983. Immunofluorescent visualization of specifically bound thrombin reveals cellular heterogeneity in number and density of preclustered receptors. *J. Cell. Physiol.* 117:297–307.
12. Carney, D. H., and J. S. Bergmann. 1982. ^{125}I-Thrombin binds to clustered receptors on noncoated regions of mouse embryo cell surfaces. *J. Cell Biol.* 95:697–703.
13. Carney, D. H., and D. D. Cunningham. 1978. Cell surface action of thrombin is sufficient to initiate division of chick cells. *Cell.* 14:811–823.
14. Carney, D. H., and D. D. Cunningham. 1978. Role of specific cell surface receptors in thrombin-stimulated cell division. *Cell.* 15:1341–1349.
15. Carney, D. H., and D. D. Cunningham. 1978. Transmembrane action of thrombin initiates chick cell division. *J. Supramol. Struct.* 9:337–350.
16. Carney, D. H., K. C. Glenn, and D. D. Cunningham. 1978. Conditions which affect initiation of animal cell division by trypsin and thrombin. *J. Cell. Physiol.* 95:13–22.
17. Carney, D. H., K. C. Glenn, D. D. Cunningham, M. Das, C. F. Fox, and J. W. Fenton II. 1979. Photoaffinity labeling of a single receptor for alpha-thrombin on mouse embryo cells. *J. Biol. Chem.* 254:6244–6247.
18. Carney, D. H., G. J. Herbosa, J. Stiernberg, J. S. Bergmann, E. A. Gordon, D. L. Scott, and J. W. Fenton II. 1986. Double signal hypothesis for thrombin initiation of cell proliferation. *Sem. Thromb. Hemostasis.* 12:231–240.
19. Carney, D. H., D. L. Scott, E. A. Gordon, and E. F. LaBelle. 1985. Neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of DNA synthesis: Role of phosphoinositides in thrombin mitogenesis. *Cell.* 42:479–488.
20. Carney, D. H., J. Stiernberg, and J. W. Fenton II. 1984. Initiation of proliferative events by human thrombin requires both receptor binding and enzymic activity. *J. Cell. Biochem.* 26:181–195.
21. Chen, L. B., and J. M. Buchanan. 1975. Mitogenic activity of blood components. I. Thrombin and prothrombin. *Proc. Natl. Acad. Sci. USA.* 72:131–135.

22. Chen, L. B., N. N. H. Teng, and J. M. Buchanan. 1976. Mitogenicity of thrombin and surface alterations on mouse splenocytes. *Exp. Cell Res.* 101:41–46.

23. Cherington, P. V., and A. B. Pardee. 1980. Synergistic effects of epidermal growth factor and thrombin on the growth stimulation of diploid Chinese hamster fibroblasts. *J. Cell. Physiol.* 105:25–32.

24. Crossin, K. L., and D. H. Carney. 1981. Evidence that microtubule depolymerization early in the cell cycle is sufficient to initiate DNA synthesis. *Cell.* 23:61–71.

25. Czech, M. P., C. L. Oppenheimer, and J. Massague. 1983. Interrelationships among receptor structures for insulin and peptide growth factors. *Fed. Proc.* 42:2598–2601.

26. Esmon, N. L., W. G. Owen, and C. T. Esmon. 1982. Isolation of membrane bound cofactor for thrombin-catalyzed activation of protein C. *J. Biol. Chem.* 257:859–864.

27. Flier, J. S., P. Usher, and A. C. Moses. 1986. Monoclonal antibody to the type I insulin-like growth factor (IGF-I) blocks IGF-1 receptor-mediated DNA synthesis: clarification of the mitogenic mechanisms of IGF-I and insulin in human skin fibroblasts. *Proc. Natl. Acad. Sci. USA.* 83:664–668.

28. Galfre, G., S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard. 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature (Lond.).* 266:550–552.

29. Glenn, K. C., D. H. Carney, J. W. Fenton II, and D. D. Cunningham. 1980. Thrombin active site regions required for fibroblast receptor binding and initiation of cell division. *J. Biol. Chem.* 255:6609–6616.

30. Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature (Lond.).* 279:679–684.

31. Gordon, E. A., and D. H. Carney. 1986. Thrombin receptor occupancy initiates cell proliferation in the presence of phorbol myristate acetate. *Biochem. Biophys. Res. Commun.* 141:650–656.

32. Gordon, E. A., J. W. Fenton II, and D. H. Carney. 1986. Thrombin receptor occupancy initiates a transient increase in cAMP levels in mitogenically responsive hamster (NIL) fibroblasts. *Ann. NY Acad. Sci.* 485:249–261.

33. Gospodarowicz, D., K. B. Brown, C. R. Bridwell, and B. R. Zetter. 1978. Control of proliferation of human vascular endothelial cells to fibroblast growth factor and thrombin. *J. Cell Biol.* 77:774–788.

34. Haigler, H., and G. Carpenter. 1980. Production and characterization of antibody blocking epidermal growth factor: receptor interactions. *Biochim. Biophys. Acta.* 598:314–325.

35. Haigler, H., J. F. Ash, S. J. Singer, and S. Cohen. 1978. Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. *Proc. Natl. Acad. Sci. USA.* 75:3317–3321.

36. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibodies of predefined specificity. *Nature (Lond.).* 256:495–497.

37. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. *Nature (Lond.).* 227:680–685.

38. Low, D. A., R. W. Scott, J. B. Baker, and D. D. Cunningham. 1982. Cells regulate their mitogenic response to thrombin through release of protease-nexin. *Nature (Lond.).* 298:476–478.

39. Low, D. A., H. S. Wiley, and D. D. Cunningham. 1985. Role of cell-surface thrombin binding sites in mitogenesis. *Cancer Cells.* Vol. 3. Growth Factors and Transformation. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 401–408.

40. Malawista, S. E., and M. C. Weiss. 1974. Expression of differentiated functions in hepatoma cell hybrids: high frequency of induction of mouse albumin production in rat hepatoma-mouse lymphoblast hybrids. *Proc. Natl. Acad. Sci. USA.* 71:927–931.

41. Maxfield, F. R., J. Schlessinger, Y. Schechter, I. Pastan, and M. C. Willingham. 1978. Collection of insulin, EGF, and alpha$_2$ macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization. *Cell.* 14:805–814.

42. Morgan, D. O., L. Ho, L. J. Korn, and R. A. Roth. 1986. Insulin action is blocked by a monoclonal antibody that inhibits the insulin receptor kinase. *Proc. Natl. Acad. Sci. USA.* 83:328–332.

43. Moss, M., H. S. Wiley, J. W. Fenton II, and D. D. Cunningham. 1983. Photoaffinity labeling of specific alpha thrombin binding sites on Chinese hamster lung cells. *J. Biol. Chem.* 258:3996–4002.

44. Murthy, U., M. Basu, A. Sen-Majumdar, and M. Das. 1986. Perinuclear location and recycling of epidermal growth factor receptor kinase: immunofluorescent visualization using antibodies directed to kinase and extracellular domains. *J. Cell Biol.* 103:333–342.

45. Pastan, I. H., and M. E. Willingham. 1981. Journey to the center of the cell: role of the receptosome. *Science (Wash. DC).* 214:504–509.

46. Perez-Rodriques, R., A. Franchi, and J. Pouyssegur. 1981. Growth factor requirements of Chinese hamster lung fibroblasts in serum-free media: high mitogenic action of thrombin. *Cell Biol. Int. Rep.* 5:347–357.

47. Pohjanpelto, P. 1977. Proteases stimulate proliferation of human fibroblasts. *J. Cell. Physiol.* 91:387–392.

48. Pohjanpelto, P. 1978. Stimulation of DNA synthesis in human fibroblasts by thrombin. *J. Cell. Physiol.* 95:189–194.

49. Raben, D. M., and D. D. Cunningham. 1985. Effect of EGF and thrombin on inositol-containing phospholipids of cultured fibroblasts: stimulation of phosphatidylinositol synthesis by thrombin but not EGF. *J. Cell. Physiol.* 125:582–590.

50. Reddan, J. R., D. C. Dziedzic, and S.J. McGee. 1982. Thrombin induces cell division in rabbit lenses cultured in a completely defined serum-free medium. *Invest. Ophthalmol. Visual Sci.* 22:485–493.

51. Schreiber, A. B., I. Lax, Y. Yarden, Z. Eshhar, and J. Schlessinger. 1981. Monoclonal antibodies against receptor for epidermal growth factor induce early and delayed effects of epidermal growth factor. *Proc. Natl. Acad. Sci. USA.* 78:7535–7539.

52. Stiernberg, J., D. H. Carney, J. W. Fenton II, and E. F. LaBelle. 1984. Initiation of DNA synthesis by human thrombin: relationships between receptor binding, enzymatic activity and stimulation of ^{86}Rb$^+$ influx. *J. Cell. Physiol.* 120:289–295.

53. Stiernberg, J., E. F. LaBelle and D. H. Carney. 1983. Demonstration of a late amiloride-sensitive events as a necessary step in initiation of DNA synthesis by thrombin. *J. Cell. Physiol.* 117:272–281.

54. Thompson, W. C., and D. H. Carney. 1984. Mitogenic responsiveness of mouse embryo cell lines with high alpha-thrombin receptor number. *J. Cell Biol.* 99:417a. (Abstr.)

55. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

56. Van Obberghen-Shilling, E., and J. Pouyssegur. 1985. Affinity labeling of high-affinity alpha-thrombin binding site on the surface of fibroblasts. *Biochim. Biophys. Acta.* 847:335–343.

57. Wall, D. A., and R. R. Hubbard. 1981. Galactose-specific recognition system of mammalian liver: receptor distribution on the hepatocyte cell surface. *J. Cell Biol.* 90:687–696.

58. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.

59. Zetter, B. R., and H. M. Antoniades. 1979. Stimulation of human vascular endothelial cell growth by a platelet-derived growth factor and thrombin. *J. Supramol. Struct.* 11:361–370.

60. Zetter, B. R., L. P. Chen, and J. M. Buchanan. 1977. Binding and internalization of thrombin normal and transformed chick cells. *Proc. Natl. Acad. Sci. USA.* 74:596–600.