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GLYCOSYLATION OF HIGH-AFFINITY THROMBIN RECEPTORS APPEARS NECESSARY FOR THROMBIN BINDING

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Summary: Monosaccharide binding competition, lectin affinity chromatography, and glycosylation inhibitors have been used to determine if glycosylation plays a role in thrombin-receptor interactions. Mannose appeared to specifically inhibit thrombin binding to mouse embryo (ME) and hamster fibroblasts. Concanavalin A bound to antibody-purified receptor fractions, and was used as an affinity ligand to purify receptor fractions that retained thrombin binding activity. Cells treated with tunicamycin (6.25 ng/ml) for 24 h lost ~ 35% of their high-affinity thrombin binding sites, yet binding of receptor monoclonal antibody TR-9 was not affected, indicating that the receptor was present in the membrane, but unable to bind thrombin. Thus thrombin receptor glycosylation may be directly involved in thrombin binding.

Although best known for cleavage of fibrinogen to form fibrin clots, thrombin initiates a number of post-clotting cellular events involved in wound healing and vascular response to injury. Many of these responses require both proteolytic activity and interaction with specific high-affinity thrombin receptors (1-3). Receptor occupancy by DIP-α-thrombin (4,5), thrombin receptor binding domain peptides (6), or receptor monoclonal antibodies (7) stimulates mitogenic signals in fibroblasts, but proteolytic cleavage or activation of protein kinase C also appear to be required (8). Consistent with this "double signal" model for mitogenic signaling (2), a functional receptor or receptor subunit has been cloned which has a unique thrombin cleavage site which activates the receptor (9).

Thrombin receptors have primarily been studied in fibroblasts which have from 1 to 8 x 10^5 sites per cell with a Kd of ~1nM (1). Receptors are clustered on the cell surface, but are not rapidly internalized (10,11). Crosslinking studies with active ^{125}I-thrombin (12) and inactivated thrombin (13,14), show receptor components of M_r = 50,250 and 150,000, respectively, and a complex with subunits of M_r = 50,000, 100,000 and 150,000 has been purified using receptor monoclonal antibody TR-9 (7). The functional thrombin receptor from Dami megakaryocytic cells, has a predicted M_r = <50,000, with 3 possible glycosylation sites (9). The Dami cell receptor may

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thus be similar to the fibroblast receptor, yet it has not been shown to possess high-affinity thrombin binding activity. This may indicate that high-affinity binding may only be achieved if all subunits are expressed, or that high-affinity binding may require specific receptor glycosylation.

In the present study, we have utilized monosaccharide competition, lectin interactions, and specific glycosylation inhibitors to determine if carbohydrate moieties play a role in the expression of thrombin receptors on the cell surface or the interaction of these receptors with α-thrombin.

MATERIALS AND METHODS

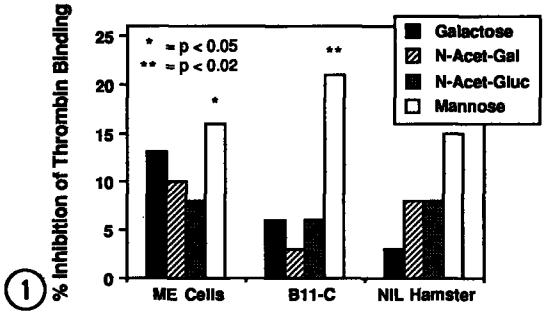
Cell Cultures: Primary cultures of mouse embryo (ME) fibroblasts were prepared from 9- to 13-day old embryos of ICR mice (15). NIL cells are an established strain of Syrian hamster fibroblasts provided by Frank Solomon. B11-C cells are an established strain of ME cells selected for high numbers of thrombin binding sites (16). Cells were grown and subcultured in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F_{12} medium (DV/F_{12}, Grand Island Biological Co., Grand Island, NY) supplemented with 10% calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml), in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were subcultured into 24-well plates (6×10^4 cell/cm^2). After 24h, medium was removed and the cells were rinsed and incubated in serum-free DV/F_{12} medium for 48h to bring the cells to a quiescent G_1/G_0 arrested state (17). In some experiments, tunicamycin was added to the medium 24 hrs after serum removal, and cells were incubated for an additional 24 h.

Membrane Extracts: Plasma membranes were prepared by lysing cells in 10 mM Tris-HCl, 1.0 mM EDTA, 0.1U/ml aprotinin, homogenization (Polytron at 35,000 RPM), and collecting plasma membranes at the 5 to 35% sucrose interface following discontinuous sucrose gradient centrifugation. Membranes were rinsed in PBS and pelleted by centrifugation (180,000 xg) and then frozen at -80°C or extracted directly with Tris buffered saline (TBS, 10 mM Tris, 150 mM NaCl, 1.0 mM $CaCl_2$), with 10 mM 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS), and 0.1 U/ml aprotinin. After 15 min, nonextracted material was removed by microcentrifugation (17,000 xg) for 10 min.

^{125}I-Thrombin Binding Assays: Purified human α-thrombin was iodinated using lactoperoxidase in the presence of benzamidine (1). Cell Binding: Specific ^{125}I-thrombin binding to ME, NIL, or B11C cells was determined with or without monosaccharides to test for binding competition as described (1). Binding to Membrane Fractions: Membrane fractions were adsorbed to nitrocellulose (using 96 well dot blot apparatus). The nitrocellulose was rinsed with TBS, blocked with 5% BSA in TBS for 1 h, and then incubated for 2 h at 23°C with 60 ng/ml ^{125}I-thrombin (in 5% BSA) in the presence or absence of a 100-fold excess of unlabeled thrombin. The nitrocellulose was then rinsed once with TBS alone, twice with 0.5% Nonidet P-40 (NP-40) in TBS, and once again with TBS. Dots corresponding to sample wells were removed and specific binding was determined as described (1).

RESULTS

Glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and mannose are major constituents of membrane glycoproteins. Therefore, these sugars were tested for competition with ^{125}I-thrombin binding to receptor sites on ME, NIL, and



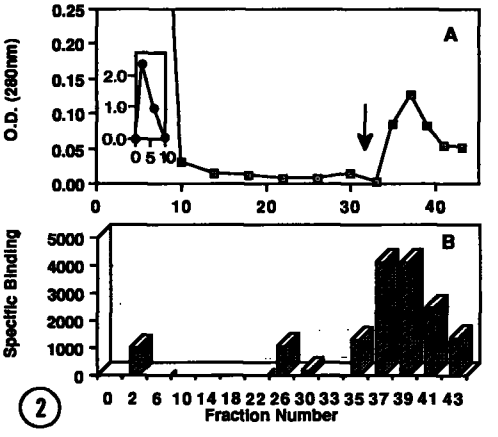
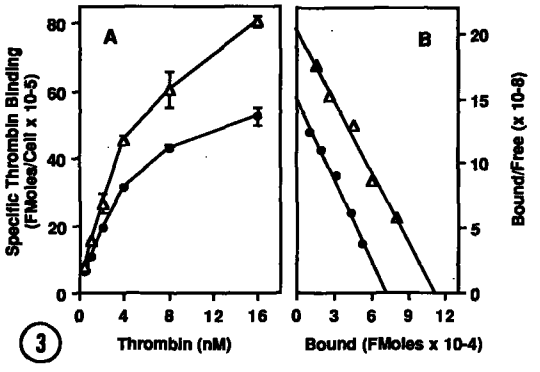


Figure 1. Inhibition of ^{125}I-thrombin binding by monosaccharides. Monolayer cultures of ME, B11-C, and NIL cells in 24-well plates were rinsed and incubated with ^{125}I-thrombin (30 ng/ml) with or without 50 mM mannose, galactose, N-acetylgalactosamine, or N-acetylglucosamine in the binding medium. P Values represent Student T tests relative to control binding without monosaccarides.

Figure 2. Binding of ^{125}I-thrombin to CHAPS-solubilized B11-C cell membranes fractionated on Concanavalin A-Sepharose. CHAPS solubilized B11-C cell membranes (see Methods) were loaded onto a 1 x 5-cm column of Concanavalin A-Sepharose, and eluted in TBS-CHAPS or TBS-CHAPS plus 100 mM α-methyl-D-mannose (at arrow). ^{125}I-thrombin binding was determined for fractions using nitrocellulose dot blots as described in Methods. Each data point is the average of O.D. 280 or specific binding determinations (CPM/25μl) from 2 adjacent fractions.

B11-C cells. Mannose (50mM), inhibited ^{125}I-thrombin binding to all 3 cell types with the greatest inhibition occuring in B11-C cells (16) which were selected for high numbers of thrombin receptors (Fig. 1). Other monosaccharides closely related in structure decreased ^{125}I-thrombin binding slightly in ME cells, but only 3-8% in NIL and B11-C cells, indicating a specific effect of mannose.

Preliminary studies showed that when TR-9 monoclonal antibody affinity-purified receptor fractions were dot-blotted onto nitrocellulose, they bound Concanavalin A, but did not bind wheat germ agglutinin (18). This suggested that the receptor itself may be glycosylated with a mannose containing oligosaccharide. and that Concanavalin A columns might be used to purify the receptor. CHAPS-solubilized membranes from 500 cm^2 cultures of B11-C cells (~4 x 10^7 cells) were therefore loaded onto a 1 x 5 cm column of Concanavalin-A-Sepharose and the runthrough and bound fractions assayed for ^{125}I-thrombin binding (Fig. 2). Although a small amount of specific ^{125}I-thrombin binding was detected in the runthrough and some fractions eluting in TBS-CHAPS, nearly 85% of the material retaining receptor specific binding activity eluted in 100 mM α–methyl-D-mannose (Fig. 2). Specific ^{125}I-α-thrombin binding to these fractions was also observed using DIP-inactivated thrombin as a noncompetitive inhibitor. Thus, the observed thrombin binding was due to thrombin interaction with receptors rather than protease-nexin or antithrombin-III which could not bind inactive thrombin.



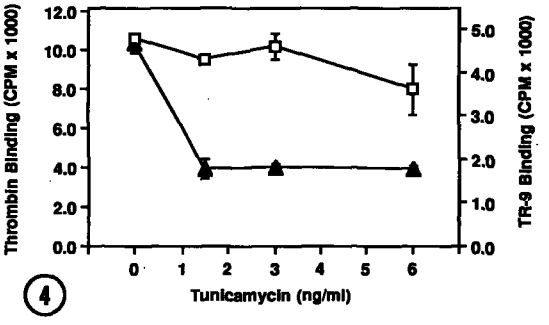


Figure 3. Effect of 24 h tunicamycin treatment on ^{125}I-thrombin binding to NIL cells. Monolayer cultures of NIL cells, treated for 24 h with 6.25 ng/ml tunicamycin (●) or control solution (Δ), were rinsed and incubated with ^{125}I-thrombin for 2 h at 23°C with and without 5 μg/ml unlabeled thrombin to determine specific thrombin binding (A). Scatchard-type analysis of binding data (B).

Figure 4. Effects of 24 h tunicamycin treatment on ^{125}I-thrombin and ^{125}I-TR-9 binding. Monolayer cultures of NIL cells treated for 24 h with tunicamycin (0 to 6 ng/ml) were rinsed and incubated with either (▲) ^{125}I-thrombin or (□)^{125}I-TR-9 monoclonal antibody (radiolabeled as thrombin, but without benzamidine) to determine specific binding to thrombin receptors.

To study the interrelationship of glycoprotein synthesis with the functioning of the thrombin receptor, NIL hamster fibroblasts were treated with different concentrations of tunicamycin. At concentrations above 25 ng/ml, tunicamycin inhibited thymidine and amino acid incorporation, but at 6.25 ng/ml, tunicamycin suppressed glycoprotein synthesis by ~30% without affecting cell viability or incorporation of amino acids or thymidine. Binding of ^{125}I-thrombin to cells treated with 6.25 ng/ml tunicamycin was 30-35% less than that of control cells at thrombin concentrations from 1 to 16nM (Fig. 3A). Scatchard-type analysis of this data suggests that the decrease is largely due to a decrease in number of functional thrombin binding sites and not a change in affinity (Fig 3B). Similar results were obtained with tunicamycin concentrations as low as 1 ng/ml and with tunicamycin treatment of B11-C cells. Tunicamycin added to cells just before measuring thrombin binding, however, had no effect. Interestingly, tunicamycin treatment did not decrease the binding of ^{125}I-labeled TR-9 monoclonal antibody to the receptor (Fig. 4). Thus it appears that the receptors are present under these conditions, but unable to bind ^{125}I-thrombin. These observations suggest that N-glycosylation may be involved with the binding of thrombin to its receptor either directly, with the sugar residues being an integral part of the thrombin binding site, or indirectly, by promoting a conformation which exposes the binding domain.

DISCUSSION

The fibroblast thrombin receptor has not yet been characterized in a purified form or sequenced to determine if it is, indeed, a glycoprotein. Recent cloning of a

functional thrombin receptor from human mesothelial cells, however, indicates potential glycosylation sites near the N- terminal tail and in the vicinity of the proposed thrombin cleavage site (9). The present studies show: i) that mannose, but not other monosaccharides compete with thrombin for receptor binding, ii) that molecules with thrombin-receptor binding activity, bind Concanavalin A Sepharose and are eluted with mannose, and iii) that inhibition of glycosylation with tunicamycin inhibits thrombin binding to cells without inhibiting antibody binding to the receptor. This suggests that thrombin binding sites are glycoproteins, with mannose residues that are essential for high-affinity interaction with thrombin.

Other growth factor receptors have been shown to be glycosylated to varying degrees. The ligand binding domain of the EGF receptor has a high content of carbohydrate (approximately 30,000 daltons), all present as N-linked oligosaccharide chains (19-22). Approximately 10-11 of the 12 potential -NXST- sites for N-glycosylation are occupied by complex, mannose-type oligosaccharide chains (21, 23).

Enzymatic deglycosylation of the mature EGF receptor does not seem to significantly change EGF binding activity (24). However, if addition of oligosaccharide chains is blocked by tunicamycin, which inhibits the first step of the pathway that leads to synthesis of N-glycoside-linked oligosaccharides (25), then the nonglycosylated receptor molecules are unable to bind EGF when assayed in intact cells or solubilized extracts (20, 26). Thus, N-glycosylation is important in the biosynthetic process of receptor activation, but the oligosaccharide chains may not be directly involved in the binding of EGF to its receptor.

Our results suggest that oligosaccarides on the high-affinity thrombin receptor may be directly involved in receptor binding. Like the EGF receptor, when oligosaccharide chain addition is blocked by tunicamycin, the binding of thrombin to intact cells is inhibited. Radiolabeled TR-9 receptor monoclonal antibody binding, however, is not inhibited by tunicamycin treatment suggesting that the receptor molecule itself is present on the surface of these cells. In addition, mannose residues compete for thrombin binding to its receptor, suggesting that mannose residues may be present near the ligand binding domain of the receptor.

That Concanavalin A, which binds high mannose glycoproteins (27), binds to thrombin receptors, suggests that the oligosaccharide chains in the thrombin receptor may be of the high-mannose type. Two additional N-glycosylation inhibitors (1-deoxynojirimycin, which blocks glucose removal from oligosaccharide chains prior to attachment of terminal sugars , and 1-deoxymannojirimycin, which inhibits removal of α-(1,2)-linked mannose residues) have been used in separate experiments with similar effects on ^{125}I-thrombin binding (data not shown). These inhibitors could affect both mannose-type and complex-type chains, however. Therefore, the nature of the carbohydrate chains will require additional studies with sufficient quantities of purified receptor to allow carbohydrate analysis.

That carbohydrate moieties appear to play a direct role in thrombin binding to cells may explain why thrombin binds to different types of cells with very different

affinities. Physiologically this may be important in allowing different cells in the vicinity of a wound to differentially respond to thrombin or partially degraded thrombin molecules at different times. The involvement of carbohydrate in the receptor binding function may also explain why thrombin appears to interact with varying affinities with so many different glycoproteins on platelets and other cells. Cells with altered carbohydrate may either bind thrombin with lower affinity or not at all. This may be the case with IIC-9 cells which have little demonstrable thrombin binding , but are mitogenically quite responsive to thrombin (28). Our analysis of these cells indicate that they have the same number of thrombin receptors as other hamster fibroblasts, but bind with a much lower affinity (Frost & Carney, in preparation). Transformed cells also have been reported to loose the ability to bind thrombin following viral, chemical, or spontaneous transformation (Reviewed in 3). Since many transformed cells have altered patterns of glycosylation, the apparent loss of binding in these cells may also be related to the alteration of carbohydrate moieties in the receptor. Further investigations on the glycosylation of the thrombin receptor and its role in thrombin binding to various cells are in progress. In addition as sufficient quantities of purified receptor become available, they will be treated with various endoglycosidases for a complete carbohydrate analysis.

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