Ca^{2+}-sensitive Binding of Thrombospondin to U937 Cells Is Due to the Formation of Calcium Precipitate in the Binding Medium

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Abstract

Thrombospondin (TSP) binds to U937 monocytic cells in a Ca^{2+}-enhancible and EDTA-inhibitable manner (Silverstein, R. L., and R. L. Nachman. 1987. *J. Clin. Invest.* 79:867–874; Silverstein, R. L., A. S. Asch, and R. L. Nachman. 1989. *J. Clin. Invest.* 84:546–552). We reproduced the results when RPMI cell culture medium, but not when HBSS was used as binding medium. Addition of 1 mM Ca^{2+} to RPMI medium increased the binding of TSP to suspended U937 cells more than eightfold; the increase was blocked by EDTA but not by heparin. Further studies showed that addition of 1 mM Ca^{2+} to RPMI medium resulted in an insoluble precipitate, which did not form when EDTA was present or when 1 mM extra Ca^{2+} was added to HBSS. TSP bound to the precipitate in a saturable and specific manner. The precipitate enhanced binding of TSP to MG63 osteosarcoma cells in a monolayer binding assay. Enhancement of binding in the monolayer assay was observed for fibronectin and vitronectin as well. Our data indicate that there is not a specific Ca^{2+}-dependent TSP receptor on U937 cell surface. Instead, the extra binding enhanced by Ca^{2+} is due to the formation of insoluble salts in the medium. (*J. Clin. Invest.* 1991. 87:171–176.) Key words: thrombospondin • GP IV • U937 cell

Introduction

Divalent cations are required for the binding of some extracellular matrix proteins to their cell surface receptors. Some of the best known examples are the binding of fibronectin (FN)[1], vitronectin (VN), and fibrinogen to integrins, a group of cell adhesion receptors that recognize the Arg-Gly-Asp (RGD) sequence in ligand proteins (1–3). In the absence of divalent cations or in the presence of EDTA, a divalent cation chelator, the binding is dramatically diminished. Recently, it was reported that thrombospondin (TSP), a glycoprotein rich in platelet α-granules and also secreted by many kinds of cultured cells, binds to U937 cells, a human monocyte tumor cell line, in a Ca^{2+}-enhancible and EDTA-inhibitable manner (4, 5). Binding is presumably via the membrane glycoprotein receptor GP IV (glycoprotein IV), since OKM5 monoclonal antibody against

GP IV has an inhibitory effect on the binding similar to EDTA (5). GP IV is also present on human platelets, monocytes, endothelial cells, and several tumor cell lines (6–8). TSP interacts with purified GP IV in vitro, and the interaction can be inhibited by EDTA, but not by RGD peptide (9). Studies by others have provided both supportive (10) and nonsupportive (11, 12) evidence for the hypothesis that GP IV is a receptor for TSP.

TSP is a heparin-binding protein, composed of three disulfide-bonded large (150-kD) identical subunits (for recent review, 13). A 25-kD heparin-binding domain, located at NH$_2$-terminal of the molecule, has been isolated and characterized (14). We have shown that cell surface heparan sulfate proteoglycan is a major mediator of the binding, endocytosis, and degradation of TSP by cells (15–18). The glycosaminoglycan chain of heparan sulfate proteoglycan interacts with the heparin-binding domain of TSP (18, 19). Heparin, which binds to TSP with a higher affinity than does heparan sulfate proteoglycan, is a potent inhibitor of TSP binding to many kinds of cells (15–18, 20).

It is intriguing that heparin enhances instead of inhibits the binding of TSP to U937 cells in the presence of 1 mM extra Ca^{2+} (4). It prompted us to repeat this experiment in order to understand the mechanism. We reproduced the results by following the published methods. But our further studies showed that addition of extra Ca^{2+} to the assay medium (RPMI cell culture medium) resulted in an insoluble calcium precipitate, which is solubilized by EDTA. We report in this paper that the effects of Ca^{2+} and EDTA on binding of TSP to U937 cells are due to the formation and solubilization of the precipitate.

Methods

Cell culture media and Ca^{2+}-free Hanks' balanced salt solution (CF-HBSS). RPMI 1640 and MEM media (Gibco Laboratories, Grand Island, NY; Cat. No. 430-1800 and 410-1500) were adjusted to pH 7.4 when prepared from powder. They were stored at 4°C for 1–3 mo without further adjustment of pH unless otherwise indicated. CF-HBSS was made by a modified manufacturer's recipe (Gibco; Cat. No. 310-4060, without calcium, glucose, and phenol red), and buffered to pH 7.4 with 10 mM Hepes.

Cell culture. U937 and MG63 cells were obtained from American Type Culture Collection, Rockville, MD. U937 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were subcultured 1:4 every 2 or 3 d. MG63 cells were maintained in MEM supplemented with 5% fetal bovine serum.

Protein purification and iodination. TSP, FN, and VN were purified from human platelet or plasma as described before (21–23). Purified TSP, FN, and VN were iodinated with ^{125}I by chloramine T method (15, 24). Free iodine was removed from the ^{125}I-VN preparation by gel filtration, and possibly denatured VN due to the labeling procedure was removed by heparin-affinity chromatography (25).

U937 cell suspension binding assay. The previously published procedures (4) were followed closely. Briefly, cells were washed once with RPMI, incubated with RPMI/0.2% BSA at 37°C for 1 h, and washed

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once more with RPMI or CF-HBSS containing 0.2% BSA. Cells were resuspended in RPMI or CF-HBSS containing 0.2% BSA, 0.5–1.0 × 10⁶ cell per sample, incubated with ¹²⁵I-TSP in a final vol of 0.2 ml. The additives were added from stock solutions (0.1 M CaCl₂; 0.25 M EDTA; 1 mg/ml heparin, all in Tris buffered saline [TBS], pH 7.4). After incubation at 4°C for 2 h with frequent inversion, the cell suspensions were centrifuged through silicone oil at 16,000 g in a tabletop microfuge (National, Z230M; BHG-Hermle, FRG) for 5 min. The tips of microfuge tubes containing cell pellets were amputated and counted in a gamma counter for cell-bound radioactivity. Cell viability under all the conditions was assessed by dye exclusion and was > 94%.

Saturation and competition binding assay. U937 cells in RPMI/BSA with or without 1 mM Ca²⁺ were incubated with ¹²⁵I-TSP and increasing amounts of unlabeled TSP (previously dialyzed into RPMI) in a final vol of 0.2 ml and processed as described above. To measure the binding of TSP to calcium precipitate and its saturability and specificity, ¹²⁵I-TSP was incubated in RPMI/BSA without cell but with 1 mM Ca²⁺ and increasing amounts of unlabeled TSP or other proteins at 4°C for 2 h; the mixture was then centrifuged in the absence of silicone oil. After the supernatants were carefully removed, the tips containing pellets were amputated and counted for radioactivity.

MG63 monolayer binding assay. Confluent monolayers in 12-well dishes were washed three times with MEM or CF-HBSS, incubated with iodinated ligand proteins in MEM/BSA or CF-HBSS/BSA for 2 h at 4°C or for 1 h at 37°C. The monolayers were then washed three times with cold media/BSA before being lysed with 1 N NaOH. The lysates were counted for cell-bound radioactivity.

Measurement of media turbidity increase. The turbidity increase in cell culture media following the addition of extra Ca²⁺ was monitored by measuring light scattering in the media on an L-format (90°) spectrofluorometer (SLM Instruments, Inc., Urbana, IL). The principle of light scattering has been described (26, and references therein). Excitation and emission wavelengths were both set at 400 nm with a slit width of 8 nm excitation, 4 nm emission. Measurements were performed in a 1-cm path length quartz cuvette at room temperature, 4°C, and 37°C, with constant magnetically controlled stirring. Each intensity value (in arbitrary units) was read at least 3 min after each aliquot of CaCl₂ stock solution (0.1 M in TBS, pH 7.4) was added, or longer until the signal was stable. In some experiments, the culture media were buffered with 15 mM Hepes and adjusted pH to 7.4 before the measurement.

Results

1 mM extra Ca²⁺ dramatically increases TSP binding to U937 cell. When 1 mM extra Ca²⁺ was added to U937 cells suspended in RPMI cell culture medium at 4°C, cell-bound TSP was increased more than eightfold (Fig. 1 *A*). This increase was inhibited by EDTA, but not by heparin. However, 5 mM EDTA only inhibited the increased part of the binding contributed by 1 mM extra Ca²⁺ in spite of the fact that RPMI medium itself contains 0.4 mM each of Ca²⁺ and Mg²⁺. Extra Mg²⁺, 1 mM, had no effect on the binding, added either alone or in combination with Ca²⁺ (data not shown).

Adding extra Ca²⁺ to RPMI produces insoluble precipitates. In control experiments, in which cells were omitted from the binding mixtures, the pellets from the samples with extra Ca²⁺ occasionally contained considerable radioactivity after centrifugation through silicone oil, suggesting that some particles were formed in the presence of extra Ca²⁺. To monitor the formation of particles, we measured the light-scattering change in the medium when extra Ca²⁺ was added. A dramatic increase in light scattering was observed whether or not TSP was present in the medium (Fig. 2). TSP did not have any appreciable effect on this turbidity increase (data not shown). Addition of Ca²⁺ to media at 4°C or 37°C increased light scattering similarly (not
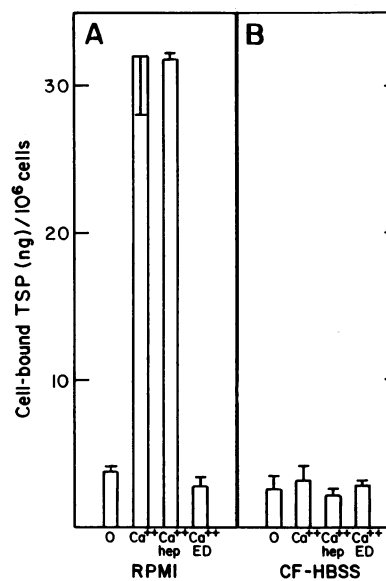

Figure 1. Binding of ¹²⁵I-TSP to suspended U937 cell in RPMI (*A*) and in CF-HBSS (*B*) media. U937 cells (0.5–1.0 × 10⁶) were incubated with ¹²⁵I-TSP (1.0 × 10⁴ cpm/μg, 0.4 μg input in *A*; 1.2 × 10⁵ cpm/μg, 0.2 μg input, normalized to 0.4 μg input, in *B*) in a final vol of 0.2 ml for 2 h at 4°C with frequent inversion. Bound and free ligands were separated by centrifugation (16,000 g, 5 min) through silicone oil, and radioactivity in pellets was quantified as cell-bound counts per minute. Each bar represents the mean value of three duplicated experiments. Error bars indicate standard deviation. Additives are indicated under the bar: *0*, no additive; *Ca⁺⁺*, 1 mM Ca²⁺; *hep*, 20 μg/ml heparin; *ED*, 5 mM (*A*) or 2.5 mM (*B*) EDTA.

shown). The solution was visibly turbid. The turbidity could be removed either by centrifugation of the medium to yield a white precipitate, or by addition of EDTA to the medium. When extra Ca²⁺ was added to a different cell culture medium, MEM, turbidity increase was also observed. There was a greater turbidity increase in the media at pH 8.3 than at pH 7.4. For RPMI, however, there was a considerable turbidity increase even at pH 7.4 when 1 mM extra Ca²⁺ was added. At this pH, 2 mM extra Ca²⁺ did not increase turbidity in MEM. Addition of Mg²⁺, up to 10 mM, had no effect on light scattering in either medium (not shown). There was no turbidity increase in CF-HBSS when up to 6 mM Ca²⁺ was added.

The Ca²⁺-enhancible and EDTA-inhibitable phenomena in TSP–U937 cell-binding assay are due to the formation and solubilization of the precipitate. The observations that extra Ca²⁺ produced insoluble precipitates in the medium that were solubilized by EDTA, and that EDTA inhibits the increase of binding contributed by extra Ca²⁺ suggested that the effects of extra Ca²⁺ and EDTA on binding of TSP to U937 cells might be due to the formation and solubilization of the precipitate. To confirm this, the suspension binding assay was performed in CF-HBSS medium, which can hold up to 6 mM extra Ca²⁺ without precipitation. If the binding of TSP to U937 cell is sensitive to Ca²⁺, then a dramatic difference would be expected when 1 mM extra Ca²⁺ is added. Instead, there was no appreciable difference with or without 1 mM extra Ca²⁺ (Fig. 1 *B*). Moreover, 2.5 mM EDTA had no inhibitory effect either.

The effects of the precipitate are not specific for TSP–U937 cell interaction. To investigate whether the effects of the precipitate are specific for TSP–U937 cell interaction, and if GP IV is involved, we tested TSP binding to MG63 cells, a human osteosarcoma cell line. These cells do not express GP IV on their surface, as assessed by immunostaining with OKM5 (a kind gift from Dr. Adam Asch, Cornell University Medical College, NY) while the U937 cells were stained positively (results not shown).
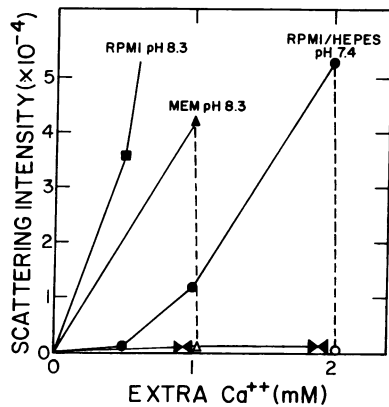
Figure 2. Light-scattering change in cell culture media after addition of extra calcium ion. The L-format spectro-fluorometer was set as described in Methods. Arbitrary units (*solid symbols*) were read at least 3 min after each aliquot of CaCl$_2$ stock solution (0.1 M in TBS) were added to 2.5 ml culture media at room temperature with constant stirring. ■, RPMI, pH 8.3; ●, RPMI/Hepes, pH 7.4; ▲, MEM, pH 8.3; ▶, MEM/Hepes, pH 7.4; ◀, CF-HBSS. The background light scattering in all media was ~100. *Dashed lines* and *open symbols* represent the light-scattering change after 5 mM EDTA was added. The scattering intensities of RPMI pH 8.3 at 1 mM Ca^{2+} and MEM pH 8.3 at 2 mM Ca^{2+} are > 1.0×10^5, thus the lines are extended off the scale.

Extra Ca^{2+}, 2 mM, enhanced binding of ^{125}I-TSP to monolayers in MEM medium (pH > 8) at 4°C, and the enhanced binding was not inhibited by heparin at a concentration that abolished the TSP binding in the absence of extra Ca^{2+} (Fig. 3 A). Indeed, heparin increased the binding nearly threefold more when added in combination with 2 mM extra Ca^{2+}. When this assay was performed in CF-HBSS, 2 mM extra Ca^{2+} did not increase the binding or reverse the inhibitory effect of heparin (Fig. 3 B). These results indicated that the effects of extra Ca^{2+} on TSP binding to MG63 cell in MEM medium were due to the presence of the precipitate.
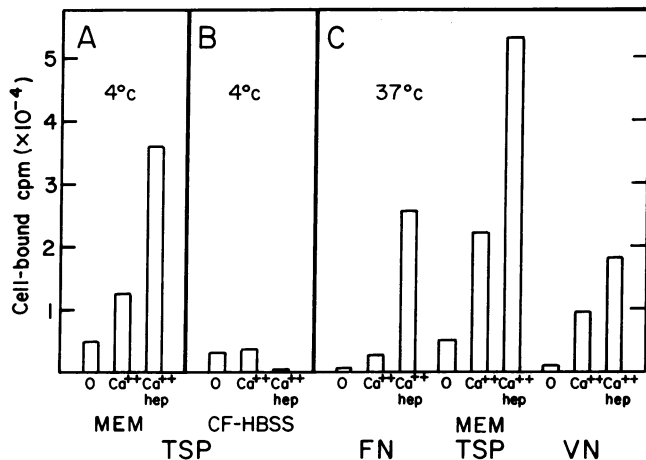

Figure 3. Effects of calcium precipitates on binding of TSP and other adhesive proteins to monolayer MG63 cell at 4°C or 37°C. Confluent MG63 monolayers in 12-well dishes were incubated with ^{125}I-TSP (1.0 × 10^5 cpm/μg, 1-μg load) in MEM/BSA (A) or CF-HBSS/BSA (B) at 4°C for 2 h, washed three times, and lysed with 1 N NaOH. The lysates were counted for cell-bound counts per minute. In C, the ^{125}I-labeled FN, TSP, and VN were adjusted to ~ 1 × 10^5 cpm/μg, 1-μg load each, and incubated in MEM/BSA with the monolayers at 37°C for 1 h. Each bar represents the mean value of duplicates. Additives are indicated under the bar: 0, no additive; Ca^{++}, 2 mM Ca^{2+}; hep, 10 μg/ml heparin. MEM contains 1.8 mM Ca^{2+}.

When ^{125}I-labeled TSP, FN, or VN were used as ligands in the MG63 monolayer-binding assay performed in MEM at 37°C, extra Ca^{2+} produced the same phenomenon (Fig. 3 C) as observed with TSP at 4°C. 2 mM extra Ca^{2+} enhanced the binding severalfold, and addition of heparin in the presence of extra Ca^{2+} further increased binding. The binding of TSP to MG63 monolayer, in MEM without extra Ca^{2+}, is inhibited by heparin, or by pretreatment of the monolayer with heparitinase (Hess, S., F. J. Fogerty, and D. F. Mosher, unpublished data), suggesting that the cell-surface heparan sulfate proteoglycan is a major binding site for TSP. The small amount of FN that binds to MG63 cell in MEM with 0.2% BSA and without extra Ca^{2+} represents largely nonspecific binding (Fogerty, F. J., W. J. Checovich, and D. F. Mosher, unpublished observations). In contrast, about 50% of the VN that binds without extra Ca^{2+} is competed by unlabeled VN, > 20 μg/ml, and thus represents specific binding (Tomasini, B. R., S. Asakura, and D. F. Mosher, unpublished observations).

TSP binds to the precipitate in a saturable and specific manner. The saturability of binding of TSP to suspended U937 cells at 4°C in the presence and absence of 1 mM extra Ca^{2+} was tested. In the absence of extra Ca^{2+}, TSP bound to U937 cell in an unsaturable manner (Fig. 4 A). If this binding was considered as "nonspecific," and the binding in the presence of extra Ca^{2+} as "total," saturable "specific" binding curve was obtained (Fig. 4 A, bottom, dashed line). The binding data were analyzed using the Ligand program of Munson and Rodbard (27) for nonlinear curve fitting, as was done in reference 4. The analysis (Fig. 4 B) best fit the model of two binding sites rather than one binding site ($F = 7.03$, $P = 0.01$). The first class of binding site had a high affinity (K_{d_1} = 0.3 nM) but low capacity (< 2% of total TSP binding). The second class of binding site had a maximum binding of 1.12 pmol TSP (in 0.2 ml binding medium with 0.54 × 10^6 cells, equivalent to 1.25 × 10^6 molecules per cell), with a K_{d_2} of 127 nM which is in accordance with the reported K_d of 145 nM (4).

When ^{125}I-TSP was incubated in RPMI/BSA without cells but with 1 mM extra Ca^{2+} and centrifuged in the absence of silicone oil, > 65% of radioactivity was recovered in pellets indicating that TSP binds to the precipitate. The binding was competed by excess unlabeled TSP (Fig. 4 C, closed circles). Ligand program analysis (not shown) gave similar K_ds (K_{d_1} = 0.16 nM, K_{d_2} = 160 nM) and increased maximum binding (B_{max1} = 0.58 pmol, B_{max2} = 9.0 pmol) compared to results obtained in the presence of cells and silicone oil. Other proteins are poor competitors. A calcium-binding human IgG (28) (Fig. 4 C) and two apatite-binding proteins, hemoglobin and ovalbumin (29) (not shown), did not compete at concentrations up to 1 mg/ml; FN at this concentration competed minimally (Fig. 4 C). Little radioactivity was recovered in the tips of microfuge tubes in the absence of extra Ca^{2+} or when incubated in CF-HBSS in the presence of 2 mM extra Ca^{2+} (not shown).

Centrifugation of ^{125}I-TSP and the precipitate through silicone oil in the absence of cells yielded inconsistent results.

Discussion

We found that addition of extra Ca^{2+} to RPMI medium resulted in a precipitate. The enhancement of TSP binding to U937 cells by extra Ca^{2+} and the inhibition of that enhancement by EDTA, as had been previously reported (4, 5) and
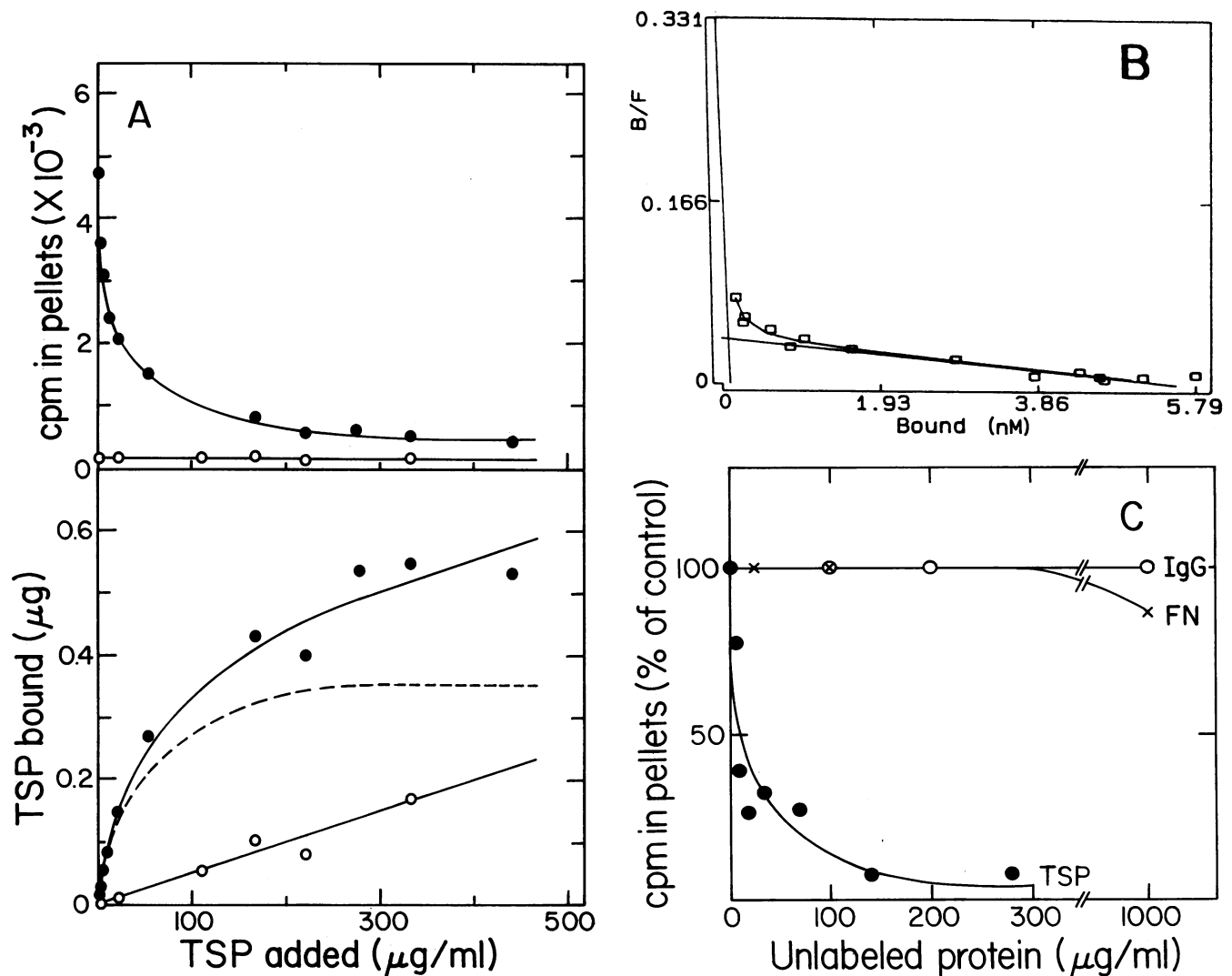
Figure 4. Saturable binding of TSP to the precipitate with or without cells. (*A*) U937 cells (0.54×10^6) in RPMI/BSA were incubated with ^{125}I-TSP (6.2×10^4 cpm) and increasing amount of unlabeled TSP in the presence (●) and absence (○) of 1 mM extra Ca^{2+}, and centrifuged through silicone oil. The same set of binding data were plotted to show the radioactivity (*top*) and total TSP (*bottom*) in the bound fractions. To calculate total bound TSP, it was assumed that labeled and unlabeled TSP behaved identically. The dashed line in the lower panel represents binding that depends on formation of the calcium precipitate. (*B*) Plot of Ca^{2+}-dependent binding as analyzed by the ligand program. Estimates of K_d and maximum binding are given in the text. (*C*) ^{125}I-TSP was incubated without cells in RPMI/BSA with 1 mM extra Ca^{2+} and in the absence (control) or presence of increasing amount of unlabeled TSP (●), or FN (×) and a human calcium-binding IgG (28) (○). The resulting precipitate was collected by centrifugation in the absence of silicone oil, and bound ^{125}I-TSP was quantified. In TSP competition experiment, 7.2×10^4 cpm was loaded and 4.8×10^4 cpm was recovered in pellets of control samples.

reproduced in our experiments, seemed to be due to formation and solubilization of the precipitate, inasmuch as (*a*) although RPMI medium itself contained 0.4 mM each of Ca^{2+} and Mg^{2+}, 5 mM EDTA only inhibited the increase of the binding contributed by 1 mM extra Ca^{2+}; (*b*) the precipitate formed in the presence of extra Ca^{2+} was solubilized by EDTA; (*c*) when the same binding assay was performed in CF-HBSS medium, in which 1 mM extra calcium did not produce a precipitate, addition of Ca^{2+} or EDTA did not change the binding; and (*d*) TSP bound to the precipitate in a saturable and specific manner in a simpler binding assay in which cells and silicone oil were omitted. Our data indicate, therefore, that the Ca^{2+}-enhancible and EDTA-inhibitable interaction is not between TSP and U937 cells, but among TSP, the precipitate, and the cells.

We found that addition of extra Ca^{2+} to MEM culture medium (pH > 8) also produced insoluble precipitates that had similar effects on MG63 monolayer binding assay. Heparin, a potent inhibitor for TSP binding to many cell lines including MG63, appreciably increased instead of inhibited the binding when added in combination with 2 mM extra Ca^{2+}. Extra Ca^{2+} did not increase the binding or reverse the inhibitory effect of heparin when the assay was performed in CF-HBSS medium, which can hold up to 6 mM extra Ca^{2+} without forming precipitate. Addition of extra Ca^{2+} to MEM also enhanced the binding of FN and VN to MG63 cells, and again, heparin further enhanced binding. We conclude, therefore, that the effects of the calcium precipitates are not specific for TSP, U937 cells, or the suspension-binding assays.

The residual binding of TSP to U937 cells in the absence of calcium precipitate was unsaturable at TSP concentrations up to 300 μg/ml[2] and was not affected by heparin or EDTA. Since it has been reported that U937 cells and human monocytes express chondroitin sulfate but not heparan sulfate proteoglycan (30, 31), these data support the hypothesis that cell surface heparan sulfate proteoglycan is an important mediator of TSP binding to cells (15–18). It was reported recently that U937 cells adhered to TSP coated substrates (32). This adhesion was inhibited by RGD-containing peptide and dependent on the presence of Ca^{2+} in the TSP coating buffer. If TSP was coated in the presence of EGTA, it did not support U937 cell adhesion. In contrast, our TSP, precoated on tissue culture dishes (Falcon 3047; Becton Dickinson Labware, Lincoln Park, NJ) at a concentration of 20 μg/ml, did not promote adhesion of U937 cells, whereas FN and fibrinogen supported adhesion when coated at concentrations as low as 0.02 μg/ml (not shown).

The binding of TSP to the mixture of precipitate and cells was saturable. The major class of binding site had a K_d very close to that previously reported (4). Our calculated maximum binding was fourfold higher than reported. This discrepancy could be explained by a difference in our experiment procedure. We dialyzed TSP into medium before it was added to the assay system. Because the RPMI medium was not diluted, more precipitate might have been produced when 1 mM Ca^{2+} was added. Thus more TSP would be in the bound fraction. Since the binding of TSP to the precipitate in the absence of silicone oil and cells had similar K_ds, it appears that TSP mainly interacted with the precipitate rather than with cells.

The precipitates produced when extra Ca^{2+} is added to the media are probably calcium phosphate as evidenced by the following pilot experiments. When $^{45}CaCl_2$ solution was added to media, radioactivity was found in the precipitate, and EDTA solubilized both precipitate and the radioactivity. Furthermore, calcium phosphate precipitates produced by addition of Ca^{2+} to PBS or commercial hydroxyapatite (Sigma Chemical Co., St. Louis, MO) produced similar phenomena in TSP–MG63 monolayer-binding assay. TSP, FN, and VN bound to the precipitates produced by addition of Ca^{2+} to culture media or PBS, as well as to commercial apatite, consistent with the fact that apatite binds many kinds of proteins (28). Immunostaining of MG63 monolayer with TSP antisera, after incubation with TSP in the presence of extra Ca^{2+}, revealed large fluorescent spots that corresponded to amorphous precipitates seen by phase microscopy. These spots were not seen in the absence of extra Ca^{2+}. Instead, staining of MG63 cells looked like the staining of Chinese hamster ovary cells reported in reference 17. This result suggests that TSP may be absorbed to the insoluble precipitates which then bind to the cell surfaces. Although the binding of TSP to MG63 monolayers was increased in the presence of extra Ca^{2+}, the degradation of TSP by the cells was decreased (< 60% of control, not shown), supporting the notion that the increased binding was not receptor-mediated, at least not via the receptor that mediates TSP degradation.

The turbidity increase in culture media is dependent on pH as well as on calcium concentration. There is a greater turbidity increase in the medium at higher pH than at lower pH. Although the pH is adjusted to 7.4 when the culture media are prepared, it gradually increases during storage (between 1 and 3 mo in our experiments) or after exposure to the air, and finally reaches a stable point between 8.3 and 8.4. Addition of 15 mM Hepes blunts but does not completely block this pH shift. Incubating the high pH (> 8) media in cell culture incubator that contains 5% CO_2 will lower the pH to physiological range, but only after several hours. We observed similar effects in TSP–MG63 cell-binding assays done in open air for 2 h (Fig. 3 A, 4°C) or in cell culture incubator containing 5% CO_2 for 1 h (Fig. 3 C, 37°C). Since most extracellular protein-cell binding assays are performed in cell culture medium and since divalent cations regulate binding of many kinds of ligands to their cell surface receptors, our studies emphasize the care that should be taken when one adds extra divalent cation to the culture media in cell-binding assays to avoid the formation of precipitate.

Binding of TSP to insoluble calcium may have physiological and pathological relevance in vivo, since TSP has been found to be deposited in calcified tissues such as bone (33) and atherosclerotic plaque (34). It was reported that TSP in activated platelet supernatant solution is adsorbed quantitatively to barium citrate precipitate while other proteins in the supernatant solution were barely adsorbed (35). Although evidence suggests that γ-carboxyglutamic acid residues are involved in adsorption to barium citrate precipitate (36), TSP contains no detectable γ-carboxyglutamic acid (35). TSP is a Ca^{2+}-binding protein (37). It has a number of potential Ca^{2+}-binding sites in its type 3 homology sequences (38). In addition, its second type 2 homology sequence probably contains a β-hydroxyasparagine (39), which has been implicated in Ca^{2+} binding to a proteolytically derived epidermal growth factor (EGF)-like domain of Factor X (40). It is not known whether or not the Ca^{2+}-binding sites are involved in the binding of TSP to the calcium precipitate, since the correlation between Ca^{2+} binding and apatite binding has not been established.

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