Thrombospondin-1 binds to polyhistidine with high affinity and specificity

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Thrombospondin-1 (TSP1) is a secreted trimeric glycoprotein of 450 kDa with demonstrated effects on cell growth, adhesion and migration. Its complex biological activity is attributed to its ability to bind to cell-surface receptors, growth factors and extracellular-matrix proteins. In this study, we used a ¹²⁵I solid-phase binding assay to demonstrate that TSP1 binds specifically to proteins containing polyhistidine stretches. Based on studies with three different six-histidine-containing recombinant proteins, we derived an average dissociation constant of 5 nM. The binding of ¹²⁵I-labelled TSP1 to these proteins was inhibited by peptides containing histidine residues, with the degree of competition being a function of the number of histidines within the peptide. Binding was not inhibited by excess histidine or imidazole, indicating that the imidazole ring is not sufficient for recognition by TSP1. Heparin was a potent inhibitor of binding

with a *K_i* of 50 nM, suggesting that the heparin-binding domain of TSP1 may be involved in this interaction. This was confirmed by the ability of a recombinant heparin-binding domain of TSP1 to directly compete for TSP1 binding to polyhistidine-containing proteins. Affinity chromatography with a polyhistidine-containing peptide immobilized on agarose revealed that TSP1 in platelet releasates is the major polypeptide retained on the six-histidine-peptide column. We conclude that TSP1 contains a high-affinity binding site for polyhistidine and this is likely to be the molecular basis for the observed binding of TSP1 to histidine-rich glycoprotein. The possibility that other polyhistidine-containing proteins also interact with TSP1 warrants further study.

Key words: extracellular matrix, heparin, histidine-rich glycoprotein.

INTRODUCTION

Thrombospondin-1 (TSP1) is a secreted glycoprotein expressed by a variety of cell types in adult and embryonic tissues, including the vascular endothelium, aortic smooth-muscle cells, megakaryocytes, monocytes and fibroblasts [1–4]. The expression of TSP1 is especially prominent at sites of tissue remodelling and injury. Consistent with this observation *in vivo*, extracellular TSP1 levels in cultured cells are tightly regulated by growth factors at the level of synthesis [5,6] and are also controlled via a novel cell-mediated degradation pathway [7,8].

Numerous biological activities have been ascribed to TSP1, including effects on platelet aggregation [9], vascular-cell proliferation [10], cell adhesion [11], cell migration [12], deposition of fibrin, and angiogenesis [13,14]. TSP1 is capable of binding to several growth factors [15–18], to a large number of extracellular-matrix proteins, including type-V collagen [19], fibrinogen [20], plasminogen [21], fibronectin [22], and histidine-rich glycoprotein (HRGP) [23], to sulphated glycolipids [24], and to several cell-surface-associated proteins including CD36 [25], integrins [26,27] and the integrin-associated protein [28]. These interactions may explain the wide variety of biological functions attributed to TSP1. However, the molecular basis for many of these interactions remains unclear. In the current study, we describe a high-affinity interaction between TSP1 and polyhistidines and have localized this binding site to the N-terminal heparin-binding domain of TSP1.

EXPERIMENTAL

Proteins and antibodies

TSP1 was purified from human platelets by adsorption to barium citrate followed by heparin–agarose chromatography, using the

methods described by Alexander and Detwiler [29]. Recombinant tumour necrosis factor-stimulated gene-6 (TSG-6), dihydrofolate reductase (DHFR) and coronin were expressed in bacteria using the vector pQE30 (Qiagen, Valencia, CA, U.S.A.). Expression of recombinant protein containing a six-histidine tag was induced in the presence of 200 nM isopropyl β-D-thiogalactoside and the cells were lysed with 8 M urea. The lysate was applied to a column of Ni²⁺-nitrilotriacetic acid resin and the proteins were eluted with 4 M urea containing 50 mM EDTA. The recombinant HBD_{1–214}, the N-terminal heparin-binding domain of TSP1, was produced using the pGEX2T vector (Pharmacia, Piscataway, NJ, U.S.A.) and purified by affinity chromatography on a GSH–Sephac column as described in [30].

Rabbit polyclonal antiserum to HBD_{1–214} was prepared by immunizing and boosting rabbits with gel slices containing the recombinant protein that had been electrophoresed on an SDS/polyacrylamide gel (10% gel) under non-reducing conditions. IgG from rabbit polyclonal anti-HBD_{1–21} was isolated by affinity chromatography on Protein G–Sephacrose.

Peptides

The three peptides used in the solid-phase binding experiments and the affinity chromatography experiments were synthesized using Fmoc (fluorenylmethoxycarbonyl) technology and an automated peptide synthesizer (Perkin Elmer, Foster City, CA, U.S.A.). Peptide 1 (MRGSHHHHHHGS), peptide 2 (GGGHHHHHHG) and peptide 3 (GGGGGGHHHG) were created by the small-scale linkage of amino acids from cartridges to an HMP (*p*-hydroxybenzyl alcohol) resin. Peptide 1 was dissolved in trifluoroacetic acid/acetic acid (1:1, v/v) and peptides 2 and 3 were dissolved in water.

Abbreviations used: TSP1, thrombospondin-1; HRGP, histidine-rich glycoprotein; TSG-6, tumour necrosis factor-stimulated gene-6; DHFR, dihydrofolate reductase; HBD_{1–214}, N-terminal heparin-binding domain of TSP1; GST, glutathione S-transferase; DTT, dithiothreitol.

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Solid-phase binding assays

Immulon 2 (Dynatech, Chantilly, VA, U.S.A.) microtitre strips with breakaway wells were coated with 3 µg/ml TSG-6, DHFR, coronin and BSA in Tris-buffered saline (pH 7.4) containing 1 mM CaCl₂ and 1 mM MgCl₂ (TBS-Ca²⁺,Mg²⁺) and incubated at 37 °C for 4 h. Non-specific sites were blocked with 3 % (w/v) BSA in TBS-Ca²⁺,Mg²⁺. Radioiodinated TSP1 (0.1–0.3 nM; specific radioactivity, 2 × 10⁵ c.p.m./µg) was added with increasing concentration of unlabelled TSP1 as competitor in TBS-Ca²⁺,Mg²⁺ with 0.05 % (v/v) Tween-20 and incubated at 4 °C overnight. The wells were washed with the same buffer and the radioactivity was determined using a gamma counter (LKB-Wallac, Turku, Finland). The competition data were analysed and dissociation constants were determined using the computer program Ligand [31]. Heterologous solid-phase binding experiments were performed in a similar manner to test the ability of various competitors to compete with polyhistidine-containing protein for binding to ¹²⁵I-TSP1. The competitors used were polyhistidine-containing peptides, HBD_{1–214}-GST (glutathione S-transferase), heparin, DNA and chondroitin sulphate.

Affinity chromatography and immunoblotting

Peptide (MRGSHHHHHHGS) synthesized at the Holland Laboratory was immobilized on to an agarose matrix column using the AminoLink Plus Coupling Gel Kit (Pierce, Rockford, IL, U.S.A.). Platelet activate was applied to the column in the presence of TBS-Ca²⁺,Mg²⁺, and rocked end-over-end for 2–4 h at 4 °C. The column was washed with TBS, and eluted with a buffer containing 10 mM Tris (pH 7.4)/0.5 M NaCl. In some cases the concentration of NaCl was increased up to 2 M and up to 100 mM EDTA was added. To analyse the proteins eluted from the peptide column, eluates were concentrated using StrataClean Resin (Stratagene, La Jolla, CA, U.S.A.). The resin–protein complexes were then resuspended in SDS sample buffer, heated to 95 °C for 5 min, and subjected to electrophoresis on 7.5 % acrylamide gels run under reducing conditions. The separated proteins were either stained with Coomassie Brilliant Blue or transferred electrophoretically on to nitrocellulose membranes (Amersham Life Sciences, Piscataway, NJ, U.S.A.) and incubated with a polyclonal antiserum directed against HBD_{1–214} and goat anti-rabbit horseradish peroxidase (Bio-Rad, Hercules, CA, U.S.A.). The bands were visualized by incubating the membranes with ECL[®] Western-blotting chemiluminescent reagent (Amersham) and exposed to Biomax MR film (Eastman Kodak, Rochester, NY, U.S.A.).

RESULTS AND DISCUSSION

TSP1 binds to polyhistidine-containing proteins with high affinity and specificity

We initiated these studies to determine whether specific binding between TSP1 and the actin-binding protein, coronin, can be demonstrated in solid-phase binding assays. The experiments were performed using ¹²⁵I-TSP1 and recombinant coronin containing six histidines proximal to the N-terminus. As shown in Figure 1, ¹²⁵I-TSP1 bound specifically to coronin since wells coated with BSA did not bind and the binding was competed for by the addition of unlabelled TSP1. However, we were surprised to find that two unrelated proteins, utilized as controls, also bound specifically to TSP1. Competition curves of all three proteins in the presence of increasing concentrations of

Figure 1 TSP1 binds specifically to six-histidine-containing proteins, TSG-6, DHFR and coronin, on solid-phase binding assay

¹²⁵I-TSP1 was added to microtitre wells coated with six-histidine-containing proteins TSG-6 (●), DHFR (■) and coronin (▲) in the presence of increasing concentrations of unlabelled TSP1 and incubated overnight at 4 °C. Following incubation wells were washed and radioactivity bound to wells was measured. BSA (▽) was used as a control and each point represents the mean ± S.D. of triplicate values. The dissociation constants for TSG-6, DHFR and coronin were determined to be 3.1, 10 and 1.5 nM, respectively, after fitting the data using single class of binding sites model and the computer program Ligand.

unlabelled TSP1 were very similar (Figure 1) and the dissociation constants for DHFR, TSG-6 and coronin were determined to be 10, 3.1 and 1.5 nM, respectively.

Sequence comparison of coronin, TSG-6 and DHFR indicated that there was no sequence similarity except for the N-terminal sequence tag containing the six histidines. Therefore we synthesized three peptides; peptide 1 containing the exact tag sequence MRGSHHHHHHGS, peptide 2 containing the sequence GGGHHHHHHG, and peptide 3 containing the sequence GGGGGGHHHG. These peptides were compared for their ability to compete for TSP1 binding to polyhistidine-containing proteins. As illustrated in Figure 2, both of the six-histidine-containing peptides were able to compete effectively for TSP1 binding to recombinant TSG-6 with *K_i* values of 82 µM for peptide 1 and 294 µM for peptide 2. By contrast, peptide 3, which contained only three histidines, was more than 20-fold less efficient than peptide 1 as a competitor, with a *K_i* value of approx. 2000 µM. In addition, we found that up to 10 mM of free histidine and imidazole were unable to inhibit the binding between TSP1 and polyhistidine-containing proteins (results not shown). The inability of free histidine or imidazole to inhibit the binding of TSP1 to polyhistidine-containing proteins supports the requirement for multiple histidines. We conclude that the ability of TSP1 to bind to three dissimilar proteins containing the six histidines is due to a specific and high-affinity interaction between TSP1 and polyhistidines that is dependent on the number of consecutive histidines.

These results probably provide the molecular basis for the previously reported interaction between TSP1 and the plasma HRGP [22,32]. Consistent with the hypothesis that HRGP binds to TSP1 via its polyhistidine sequence is that an apparent binding

Figure 2 Polyhistidine peptides can compete for binding of TSP1 with six-histidine proteins

Heterologous displacement assay demonstrates that the ability of ^{125}I -TSP1 to bind six-histidine-containing protein could be inhibited by polyhistidine-containing synthetic peptides MRGSHHHHHHGS (●; peptide 1), GGGHHHHHHG (■; peptide 2) and GGGGGGHHHG (▲; peptide 3). The values represent means $\pm$ S.D. of triplicates. The inhibition constants (K_i) for peptides 1, 2 and 3 were determined to be 82, 294 and 2000 μM , respectively, after fitting the data using single class of binding sites model and the computer program Ligand.

Figure 3 TSP1 from platelet activate can be purified by polyhistidine affinity chromatography

Platelet-activate polypeptides affinity selected on MRGSHHHHHHGS (peptide 1) agarose were eluted with 10 mM Tris buffer (pH 7.4) containing 0.5 M NaCl and 100 mM EDTA in 3.5 ml fractions. From each eluted fraction (fractions 1–6), 1 ml was concentrated using strataclean resin and subjected to electrophoresis on a 7.5% acrylamide gel under reducing conditions and stained with Coomassie Brilliant Blue (A) or immunoblotted with TSP1 polyclonal antibodies (B). Lane 1, platelet activate; lane 2, flow-through from affinity column; lanes 3–8, eluted fractions 1–6 concentrated with strataclean resin.

K_d of 7 nM was determined in the previous study [22] and this is remarkably similar to the 5 nM average K_d derived here. It is interesting to note that there are a number of other proteins with polyhistidine stretches in their sequences that may serve as potential TSP1-binding sites [33–35]. For example, an integrin-like surface protein on *Candida albicans* contains a stretch of six histidines on its extracellular domain [35]. This protein is believed to be responsible for cell binding and invasion by *C. albicans*.

Figure 4 Optimal TSP1 binding to polyhistidine requires Ca^{2+} and the presence of disulphide bonds

^{125}I -TSP1 binding to immobilized six-histidine-containing protein was carried out in the presence of increasing concentrations of DTT (▲), EDTA (■) and Ca^{2+} (●). DTT and EDTA inhibited ^{125}I -TSP1 binding to TSG-6 with IC_{50} values of 1 and 5 mM, respectively. The data are expressed as the amount of radioactivity bound over the total activity added per well and represent averaged values from duplicate samples.

Given that TSP1 is found at the dermal and epidermal junction of skin [4] and its expression is induced by injury, it is possible that TSP1 can facilitate the binding and invasion process by interacting with the polyhistidine stretch on this integrin-like protein. The possibility that high-affinity binding of TSP1 contributes to the biological activity of polyhistidine-containing proteins warrants further study.

TSP1 released from activated platelets can bind specifically to a polyhistidine-containing peptide column

TSP1 is a major component of platelet α -granules and is released upon platelet activation to facilitate tissue repair and possibly platelet aggregation. Having demonstrated that purified TSP1 can bind to polyhistidine-containing proteins in solid-phase binding assays, we next determined whether, within the complex milieu of the platelet releasate, TSP1 could bind specifically to a polyhistidine-containing peptide. Activated platelet releasate was incubated with peptide 1 immobilized on an agarose matrix, washed with buffer to remove non-specific binding and eluted with 0.5 M NaCl-containing buffer. The elution fractions, analysed by SDS/PAGE, are shown in Figure 3 and demonstrated that the 180 kDa TSP1 in activated platelet lysate (lane 1) is no longer present in the flow-through (lane 2). The 0.5 M NaCl-eluted fractions contained primarily a 180 kDa polypeptide (Figure 3A, lanes 3–8), suggesting that TSP1 retained on the polyhistidine peptide column was released in the presence of high salt. Increasing the NaCl concentration up to 2 M did not alter the rather broad elution profile substantially (results not shown).

Immunoblotting experiments with polyclonal TSP1 antiserum confirmed that the 180 kDa band is indeed TSP1 (Figure 3B). Immobilized peptide 2 containing six histidines bound TSP1 equally well and, consistent with the solid-phase binding data, the peptide containing only three histidines was greatly impaired

Figure 5 TSP1 binding to polyhistidine involves N-terminal HBD

¹²⁵I-TSP1 binding to immobilized polyhistidine-containing protein was carried out in the presence of increasing concentrations of (A) heparin; (B) heparin (●), DNA (▼) and chondroitin sulphate (○); and (C) GST-HBD₁₋₂₁₄. Each point represents the mean of triplicate values (± S.D.), data were analysed by the program Ligand and the curves represent the best fit to a model of a single class of binding sites.

in its ability to retain TSP1 (results not shown). Our data indicate that TSP1 released from activated platelets can bind specifically to polyhistidine-containing polypeptides and suggest that this interaction can occur in a physiologically relevant setting. Furthermore, we have demonstrated that polyhistidine-affinity chromatography is a novel and potentially useful method for the rapid purification of TSP1. Given that recombinant proteins containing six histidines are frequently used in biological studies, the presence of TSP1 in the assay system should be evaluated as a possible confounding factor.

Characterization of TSP1 binding to polyhistidine-containing proteins and localization of the binding site to the heparin-binding domain

As a first step towards understanding the molecular basis for the binding of TSP1 to polyhistidines, we examined the ability of calcium, EDTA and the reducing agent dithiothreitol (DTT) to modify binding activity. Solid-phase binding assays were performed in the presence of increasing concentrations of calcium, EDTA and DTT. As shown in Figure 4, DTT and EDTA inhibited ¹²⁵I-TSP1 binding to polyhistidine-containing protein with IC₅₀ values of 1 and 5 mM, respectively. By contrast, calcium had a slight positive effect on the binding of TSP1 to polyhistidine at physiologically relevant concentrations of 0.5–10 mM and inhibition of binding was not observed until 50 mM calcium was present in the incubation solution. Since both reducing agents and EDTA are known to alter the conformation of TSP1 [36–40], our data indicate that retention of some elements of the native conformation of TSP1 is critical for its ability to bind to polyhistidine.

TSP1 is a known heparin-binding protein and the portion of the TSP1 molecule found to be largely responsible for heparin binding is localized to the N-terminal domain of the protein [41–43]. As shown in Figure 5(A), we found that heparin was able to compete effectively for TSP1–polyhistidine binding in the solid-phase binding assay, with a K_i of 50 nM. Other negatively charged molecules, including chondroitin sulphate and DNA, were unable to inhibit binding, even at concentrations of 10 μM (Figure 5B). The inability of charged moieties such as chondroitin sulphate, DNA and up to 10 mM lysine (results not shown) to inhibit the binding of TSP1 to polyhistidine-containing molecules

strongly suggests that the interaction is not only based on charge. Indeed, under the conditions of binding (TBS, pH 7.4), less than 10% of the histidines should be protonated. These results suggest that heparin blocks TSP1 binding to polyhistidine-containing proteins via its known interaction with HBD of TSP1. To directly test the hypothesis that the HBD of TSP1 is responsible for polyhistidine binding, we examined the ability of a recombinant HBD of TSP1, encompassing amino acids 1–214 of TSP1 (HBD₁₋₂₁₄), to compete for TSP1 binding to polyhistidine-containing proteins. The results, shown in Figure 5(C), indicated that recombinant GST-HBD₁₋₂₁₄ was indeed able to compete effectively with ¹²⁵I-TSP1 binding to the polyhistidine-containing protein and inhibited TSP1 binding with an approximate K_i of 200 nM. Further delineation of the amino acid sequences on TSP1 involved in the high-affinity binding to polyhistidine will provide additional mechanistic insight into the nature of this binding.

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REFERENCES

- 1 Iruela-Arispe, M. L., Liska, D. J., Sage, E. H. and Bornstein, P. (1993) *Dev. Dyn.* **197**, 40–56
- 2 O'Shea, K. S. and Dixit, V. M. (1988) *J. Cell Biol.* **107**, 2737–2748
- 3 O'Shea, K. S., Rheinheimer, J. S. and Dixit, V. M. (1990) *J. Cell Biol.* **110**, 1275–1283
- 4 Wight, T. N., Raugi, G. J., Mumby, S. M. and Bornstein, P. (1985) *J. Histochem. Cytochem.* **33**, 295–302
- 5 Donoviel, D. B., Amacher, S. L., Judge, K. W. and Bornstein, P. (1990) *J. Cell Physiol.* **145**, 16–23
- 6 Framson, P. and Bornstein, P. (1993) *J. Biol. Chem.* **268**, 4989–4996
- 7 Mikhailenko, I., Kounnas, M. Z. and Strickland, D. K. (1995) *J. Biol. Chem.* **270**, 9543–9549
- 8 Godyna, S., Liao, G., Popa, I., Stefansson, S. and Argraves, W. S. (1995) *J. Cell Biol.* **129**, 1403–1410
- 9 Leung, L. L. (1984) *J. Clin. Invest.* **74**, 1764–1772
- 10 Majack, R. A., Goodman, L. V. and Dixit, V. M. (1988) *J. Cell Biol.* **106**, 415–422
- 11 Lawler, J., Weinstein, R. and Hynes, R. O. (1988) *J. Cell Biol.* **107**, 2351–2361
- 12 Tarabotti, G., Roberts, D. D. and Liotta, L. A. (1987) *J. Cell Biol.* **105**, 2409–2415

- 13 Iruela-Arispe, M. L., Bornstein, P. and Sage, H. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5026–5030
- 14 Nicosia, R. F. and Tuszyński, G. P. (1994) *J. Cell Biol.* **124**, 183–193
- 15 Murphy-Ullrich, J. E., Schultz-Cherry, S. and Hook, M. (1992) *Mol. Biol. Cell* **3**, 181–188
- 16 Schultz-Cherry, S., Ribeiro, S., Gentry, L. and Murphy-Ullrich, J. E. (1994) *J. Biol. Chem.* **269**, 26775–26782
- 17 Taraboletti, G., Belotti, D., Borsotti, P., Vergani, V., Rusnati, M., Presta, M. and Giavazzi, R. (1997) *Cell Growth Differ.* **8**, 471–479
- 18 Hogg, P. J., Hotchkiss, K. A., Jimenez, B. M., Stathakis, P. and Chesterman, C. N. (1997) *Biochem. J.* **326**, 709–716
- 19 Mumby, S. M., Raugi, G. J. and Bornstein, P. (1984) *J. Cell Biol.* **98**, 646–652
- 20 Leung, L. L. and Nachman, R. L. (1982) *J. Clin. Invest.* **70**, 542–549
- 21 Silverstein, R. L., Leung, L. L. K., Harpel, P. C. and Nachman, R. L. (1984) *J. Clin. Invest.* **74**, 1625–1633
- 22 Plow, E. F., McEver, R. P., Collier, B. S., Woods, Jr, V. L., Marguerie, G. A. and Ginsberg, M. H. (1985) *Blood* **66**, 72–74
- 23 Leung, L. L. K., Nachman, R. L. and Harpel, P. C. (1984) *J. Clin. Invest.* **73**, 5–12
- 24 Roberts, D. D., Havestick, D. M., Dixit, V. M., Frazier, W. A., Santoro, S. A. and Ginsburg, V. (1985) *J. Biol. Chem.* **260**, 9405–9411
- 25 Asch, A. S., Tepler, J., Silbiger, S. and Nachman, R. L. (1991) *J. Biol. Chem.* **266**, 1740–1745
- 26 Lawler, J. and Hynes, R. O. (1989) *Blood* **74**, 2022–2027
- 27 Smith, J. W. and Cheres, D. A. (1990) *J. Biol. Chem.* **265**, 2168–2172
- 28 Gao, A. G., Lindberg, F. P., Finn, M. B., Blystone, S.D., Brown, E. J. and Frazier, W. A. (1996) *J. Biol. Chem.* **271**, 21–24
- 29 Alexander, R. J. and Detwiler, T. C. (1984) *Biochem. J.* **217**, 67–71
- 30 Mikhailenko, I., Krylov, D., Argraves, K. M., Roberts, D. D., Liao, G. and Strickland, K. D. (1997) *J. Biol. Chem.* **272**, 6784–6791
- 31 Munson, P. J. and Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- 32 Silverstein, R. L., Leung, L. L., Harpel, P. C. and Nachman, R. L. (1985) *J. Clin. Invest.* **75**, 2065–2073
- 33 Hofmann, S. L., Topham, M., Hsieh, C. L. and Francke, U. (1991) *Genomics* **9**, 656–669
- 34 Chee, M. S., Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Horsnell, T., Hutchison, 3rd, C. A., Kouzarides, T., Martignetti, J. A. et al (1990) *Curr. Top. Microbiol. Immunol.* **154**, 125–169
- 35 Gale, C., Finkel, D., Tao, N., Meinke, M., McClellan, M., Olson, J., Kendrick, K. and Hostetter, M. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 357–361
- 36 Dixit, V. M., Galvin, N. J., O'Rourke, K. M. and Frazier, W. A. (1986) *J. Biol. Chem.* **261**, 1962–1968
- 37 Lawler, J., Chao, F. C. and Cohen, C. M. (1982) *J. Biol. Chem.* **257**, 12257–12265
- 38 Sun, X., Skorstengaard, K. and Mosher, D. F. (1992) *J. Cell Biol.* **118**, 693–701
- 39 Lawler, J., Ferro, P. and Duquette, M. (1992) *Biochemistry* **31**, 1173–1179
- 40 Prochownik, E. V., O'Rourke, K. and Dixit, V. M. (1989) *J. Cell Biol.* **109**, 843–852
- 41 Dixit, V. M., Grant, G. A., Santoro, S. A. and Frazier, W. A. (1984) *J. Biol. Chem.* **259**, 10100–10105
- 42 Aiken, M., Ciagłowski, R. E. and Walz, D. A. (1986) *Arch. Biochem. Biophys.* **250**, 257–262
- 43 Raugi, G. J., Mumby, S. M., Ready, C. A. and Bornstein, P. (1984) *Thromb. Res.* **36**, 165–175

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