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UROMODULIN (TAMM-HORSFALL PROTEIN) IS A LEUKOCYTE ADHESION MOLECULE

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Uromodulin (Tamm-Horsfall protein), the most abundant constituent of human urine, is synthesized exclusively in the kidney tubular epithelium and its amino acid sequence suggests a capacity for cell adhesion. We investigated adhesion between human uromodulin and neutrophils by allowing uromodulin, immobilized on microtiter plates, to interact with neutrophils. It was found that neutrophils attached to uromodulin in a saturable manner. The binding was inhibited by uromodulin in solution. It required metabolically active cells, was calcium sensitive and could be inhibited by arginine-glycine- aspartate-containing peptides in solution. These data suggest that uromodoulin can act as a specific ligand for neutrophils. This interaction is potentially important in leukocyte trafficking in the kidney and in the pathogenesis of interstitial nephritis.

Uromodulin (Tamm-Horsfall protein) is the most abundant protein of normal human urine (1). Its cDNA sequence (2-4) reveals the presence of four epidermal growth factor (EGF)-like motifs and one arginine-glycine-aspartate (RGD) sequence, suggesting a capacity for cell adhesion.

While the physiological function of this protein in normal kidney remains unclear, uromodulin has been implicated in the pathogenesis of experimental and clinical tubulo-interstitial nephritis (5-8). In vitro, uromodulin has been shown to activate neutrophils (9,10) and monocytes (11, 12). Direct binding between uromodulin and neutrophils has been examined in one study (13) which showed specific binding between monomeric uromodulin in solution and neutrophils at 4°C.

In the mammalian kidney, uromodulin is expressed abundantly and exclusively on the surface of the epithelial cells lining the thick ascending limb of the loop of Henle (TAL, 14), where it exists as a high molecular weight polymeric gel (MW ~ $7x10^7$ kDa). In this study, we examined binding mechanisms between neutrophils and immobilized, polymeric uromodulin, mimicking its *in - vivo* state more closely and found that neutrophils adhered to microtiter plates coated with uromodulin by a calcium dependent, RGD-mediated mechanism, requiring metabolically active cells.

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Abbreviations: ALB: human serum albumin; BSA: bovine serum albumin; EDTA: ethylenediaminetetraacetic acid; EGF: epidermal growth factor; EGTA: ethylene glycol-bis (ß-amino ethyl ether) N,N,N',N'-tetraacetic acid; HBSS: Hank's balanced salt solution; ICAM-1: intercellular cell adhesion molecule - 1; RGD: arginine-glycine-aspartic acid; RT: room temperature; TAL: thick ascending limb of loop of Henle.

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MATERIALS AND METHODS

Isolation of Uromodulin: Uromodulin was purified from human urine by minor modifications of the original salt precipitation method of Tamm and Horsfall (15), dialyzed against water, lyophilized and confirmed to be pure by the appearance of a single band on SDS-PAGE at 80 kDa. There was no band in the region of 150 - 200 kDa, excluding major contamination from IgG, another protein of large molecular weight found in urine (16).

Isolation of Human Neutrophils: Human neutrophils were isolated from heparinized whole blood by density gradient centrifugation in a Ficoll-Hypaque based medium (Mono-poly resolving medium, Flow Laboratories, McLean,VA). Contaminating erythrocytes were removed by hypotonic lysis in water. Preparations were judged to be pure on the basis of their microscopic morphology. The neutrophils were counted using a hemocytometer.

Binding Assay: 96 well polystyrene microtiter plates (Immulon 4, Dynatech, Chantilly,VA) were coated with uromodulin (100 µl of 100 µg/ml per well) or other proteins used as negative or positive controls, in bicarbonate buffer, pH 10.0, and allowed to incubate overnight at 4 °C in a humidity chamber. All experiments were done in triplicate and repeated at least once. Next morning, unbound protein solution was flicked off the plates and the plates were blocked with 1% bovine serum albumin (BSA) in Hank's balanced salt solution (HBSS, Cat.# 310-4065AF, Gibco-BRL, Gaithersburg, MD) for one hour at RT to inhibit non-specific binding. Neutrophils isolated above were then added to the wells at a concentration of 200,000 cells per well and allowed to incubate with the protein bound on the plate for 60 minutes at RT. RT was chosen because binding appeared to require metabolically active cells. 37 °C was not chosen because at 37 °C neutrophils tend to become non-specifically sticky. In some experiments, neutrophils were first incubated with a competitor of binding for 30 min at 4 °C. The wells were washed twice with 0.1% BSA in HBSS and examined under a microscope. Bound cells were quantitated using a myeloperoxidase assay described below. Human serum albumin (ALB, 100 ul of 100 µg/ml) was used as negative control and P-selectin (GMP-140, PADGEM, 100 ul of 5 µg/ml, a gift from Dr. Rodger McEver), a molecule previously shown to bind neutrophils (17), was used as a positive control.

Myeloperoxidase Assay:Neutrophils bound to the microliter plates were solubilized by incubation with 200 µl per well of 0.5% hexadecyltriethylammonium bromide in 50 mM potassium phosphate, pH 6.0, for 30 minutes at room temperature to release myeloperoxidase activity into solution. 20 µl of the above extract was added to 15 µl of 0.08 M potassium phosphate buffer, pH 6.4, 20 µl of 0.003% H_2O_2 (1:10,000 dilution of 30% H_2O_2 in the potassium phosphate buffer) and 10 µl of 16 mM tetramethylbenzidine substrate solution in N, N- dimethylformamide. The blue color reaction was stopped after 5 minutes with 100 µl of 1 M H_3PO_4, pH 3.0. The optical intensity of color was read in a spectrophotometer (Dynatech) at 450 nM. A standard curve was obtained simultaneously for each experiment by placing known number of neutrophils in sequentially increasing amounts, in triplicate, in separate wells.

Peptide Synthesis: Peptides were synthesized by the Fmoc (N^a-9-Fluorenylmethyloxycarbonyl) solid phase synthesis strategy (18) by the Molecular Biology Resource Facility of the Warren Medical Research Institute. Crude peptide mixture was separated by reverse phase C18 HPLC. All major peaks were collected and analyzed for their amino acid content following hydrolysis in 6 N HCl for 20-24 hrs. under vaccum by cation exchange chromatography. The amino acid composition was found to be within 5 % of that expected.

RESULTS

Neutrophils bound to uromodulin and to P-selectin (positive control) but not to albumin (negative control) (Fig. 1). Neutrophils preincubated with uromodulin in solution did not bind albumin (Fig. 2) suggesting that neutrophils were binding to uromodulin and not being activated by uromodulin to become non-specifically sticky. The binding of neutrophils to immobilized uromodulin was reduced by uromodulin in solution at 100 µg/ml (Fig. 3) but higher concentrations of uromodulin did not produce additional inhibition, possibly because of tendency of uromodulin to come out of solution above 100 µg/ml. A time curve revealed that neutrophil binding to uromodulin plateaus at 30 minutes. A dose-response curve, obtained by incubating equal number

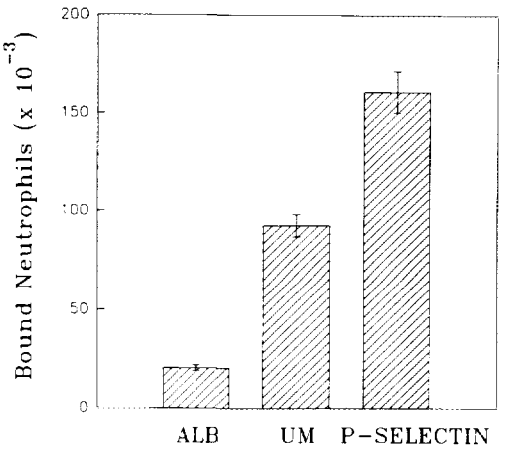
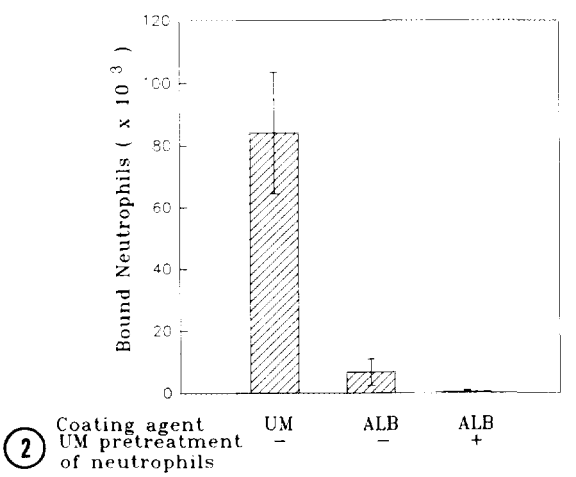


Fig. 1. **Neutrophils bind to uromodulin and P-selectin (positive control) but not to albumin (negative control).** Microtiter plates were coated with human serum albumin (ALB), 10 µg/well, uromodulin (UM), 10 µg/well and P-selectin, 0.5 µg/well, as described under methods. Freshly isolated human neutrophils (200,000/well) were allowed to incubate with them for 30 min at RT and binding measured as described under methods. Results are presented as mean ± s. d. (n=3).



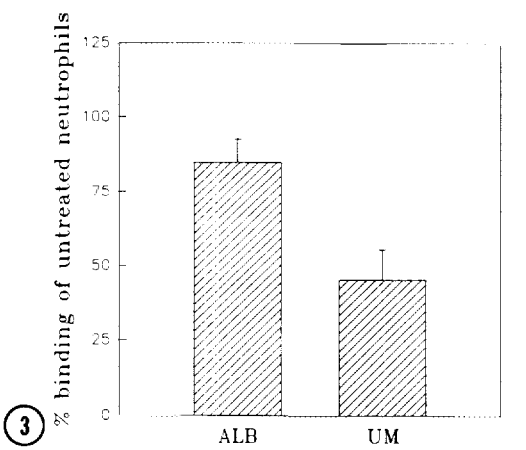


Fig. 2. **Neutrophils pre-incubated with uromodulin do not bind to albumin.** Neutrophils were allowed to incubate with wells coated with uromodulin (UM) or albumin (ALB) as described for Fig. 1. In addition, one set of neutrophils were first pretreated with uromodulin in HBSS at 50 µg/ml for 30 min at 37 °C and then allowed to incubate with uromodulin-coated wells. Results are presented as mean ± s. d. (n=3).

Fig. 3. **Neutrophil binding to immobilized uromodulin is reduced by uromodulin in solution.** Neutrophil binding to uromodulin coated microtiter plates was carried out as described for Fig. 1. In addition in some wells uromodulin (UM) or albumin (ALB, negative control) was added to neutrophil suspension at concentrations of 100 µg/ml (10 µg/well) to act as potential competitors for the immobilized UM. Results are presented as percentage of binding of untreated neutrophils (mean + s.e.m., n=9, t test, ALB vs. UM, $p < 0.007$).

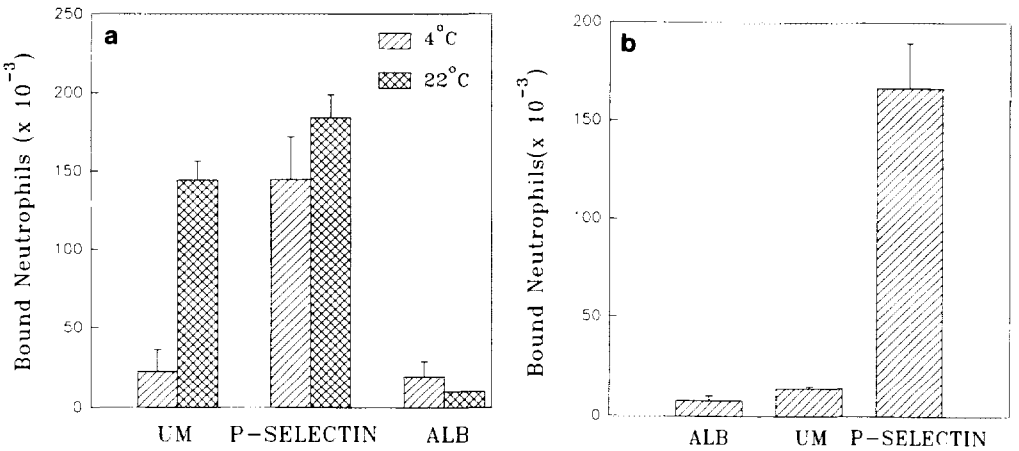


Fig. 4. **Neutrophil binding to uromodulin requires metabolically active cells.** **a)** Microtiter plates were coated with uromodulin (UM), P-selectin or albumin (ALB) and neutrophils allowed to react with them as described for Fig. 1. Parallel experiments were performed at 4 °C and at RT (22 °C). Neutrophils bind to UM at 22 °C but not at 4 °C; to P-selectin (positive control) both at 4 °C and at 22 °C; and to albumin (ALB, negative control) neither at 4 °C nor at 22 °C. **b)** Microtiter plates were coated with uromodulin (UM), P-selectin or albumin (ALB) as described for Fig. 1. Neutrophils were fixed by incubation with freshly prepared 1% paraformaldehyde in PBS, pH 7.4, for 20 min at RT. Paraformaldehyde was inactivated by adding 0.1 volume of buffer containing 200 mM Tris and 400 mM glycine buffer for 10 min. Neutrophils were then washed thrice in HBSS and allowed to incubate with microtiter plates coated with UM, P-selectin and ALB as for Fig. 1. Formalin fixed neutrophils were found not to bind uromodulin or albumin but did bind to p-selection. Results are presented as mean ± s. d. (n=3).

of neutrophils with sequentially increasing amounts of uromodulin, showed that the binding plateaus at 5 μg/well.

Neutrophil binding to lectins such as P-selectin, has been shown not to require metabolically active cells (17) but neutrophil binding to integrin ligands such as ICAM-1, is seen only in cells that are active metabolically, and is not seen in fixed or quiescent cells (19). Fig. 4 shows the need for metabolic activity in neutrophil-uromodulin interaction. Neutrophils bound to uromodulin at 22 °C but not at 4 °C (Fig. 4a). Moreover, formalin fixation of neutrophils abolished their binding to uromodulin but not to P-selectin (Fig. 4b). Together, these data demonstrate that uromodulin-neutrophil interaction in this experimental model requires metabolically active cells and resembles an integrin mediated process.

Since several cell adhesion molecules have been demonstrated to bind to their ligands in a divalent ion dependent mechanism, we next proceeded to test whether the binding of neutrophils to uromodulin was dependent upon divalent cations. Binding of neutrophils to uromodulin was determined in the presence of EDTA (chelates calcium and magnesium) or EGTA (chelates calcium but not magnesium) and both agents inhibited the binding, consistent with the calcium dependence of the binding (Fig. 5).

Integrins are dimeric protein molecules that bind specific ligands (20). Most, though not all, integrin ligands have a specific tripeptide, 'RGD' sequence. The human uromodulin molecule has one 'RGD' sequence at position 142-144, flanked by tyrosine (Y) on the 5' end and glycine

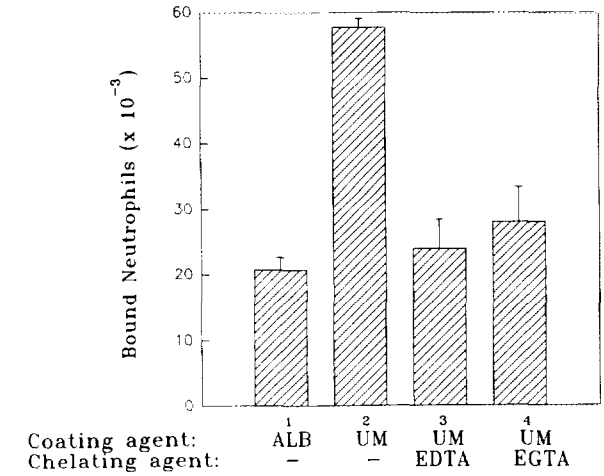


Fig. 5. **Neutrophil binding to uromodulin is calcium dependent.** Microtiter plates were coated with uromodulin (UM) and albumin (ALB) as for Fig. 1. 200,000 neutrophils suspended in HBSS were incubated with each well. In some wells 5 mM EDTA or EGTA were added to neutrophil suspension to chelate calcium and magnesium (EDTA) and calcium alone (EGTA). The binding is inhibited equally by chelation of calcium alone (EGTA) or by chelation of calcium and magnesium (EDTA). Results are presented as mean ± s. d. (n=3). ANOVA 1 vs. 2, $p < 0.05$; 2 vs. 3, $p < 0.05$; 2 vs. 4; $p < 0.05$.

(G) on the 3' end. We next tested whether this sequence 'YRGDG' was critical to the binding of neutrophils to uromodulin by examining the ability of synthetic peptides to inhibit neutrophil binding to uromodulin. Fig. 6 shows the results of these experiments and reveals that the binding was inhibited by RGD-containing pentapeptide 'YRGDG' but not by control peptides 'YRGEG'

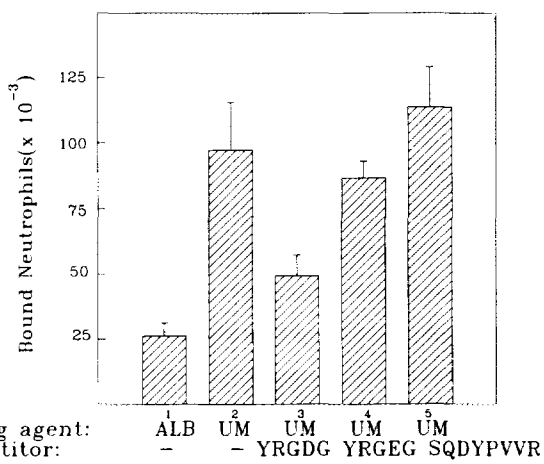


Fig. 6. **Neutrophil binding to uromodulin is "RGD" dependent.** Microtiter plates were coated with uromodulin (UM) and albumin (ALB) and neutrophils allowed to interact with them as for Fig. 1. In some wells, 'RGD' and control peptides were added to the neutrophil suspension at a concentration of 1,000 µg/ml. The binding is inhibited by 'RGD' containing peptide 'YRGDG' but not by control peptides 'YRGEG' and 'SQDYPVVR'. Results are presented as mean ± s. d. (n=3). ANOVA 1 vs. 2, $p < 0.05$; 2 vs. 3, $p < 0.05$; 2 vs. 4, $p > 0.05$; 2 vs. 5, $p > 0.05$.

and 'SQDYPVVR' suggesting not only that binding requires the 'RGD' site but also emphasizing the critical importance of the aspartic acid (D) residue in this interaction.

DISCUSSION

This study has examined interactions between polymeric immobilized uromodulin and neutrophils. Results suggest that neutrophils show saturable, reversible, time-dependent and dose-dependent binding to uromodulin by a process that requires metabolically active cells, is calcium dependent and is RGD-mediated.

These data do not directly identify the neutrophil adhesion molecule for uromodulin but do point toward potential candidate molecules. Major adhesion molecules on the neutrophil surface are L-selectin and ß2-integrins. L-selectin is constitutively expressed and binds to sialylated oligosaccharides in a lectin like interaction (21). Binding of selectins to their ligands in general does not require metabolically active cells. ß2-integrins are expressed on the neutrophil surface in an activation dependent manner and require metabolically active cells for binding. Interactions of ß2-integrins with their ligands usually requires calcium (22) and may (23) or may not (24) involve the 'RGD' tripeptide in the ligand. Our data suggest a potential role for neutrophil integrins in the binding of neutrophils to uromodulin and also provide the first evidence, to our knowledge, that the 'RGD' sequence in uromodulin is functionally active. Further studies are needed to confirm and dissect the role of individual neutrophil integrins in this interaction. Although, uromodulin has long being implicated in pathogenesis of tubulointerstitial nephritis (5-8), direct interactions between uromodulin and neutrophils have been examined only recently (9,10, 13). Horton et. al. (9) first demonstrated that particulate uromodulin stimulates respiratory burst, degranulation and leukotriene production in neutrophils. Yu et. al. (10) showed that uromodulin increases phagocytosis, complement receptor expression and arachidonic acid metabolism in neutrophils. The mechanism of binding between neutrophils and uromodulin has been examined in only one other study (13). Thomas et. al. (13) used radiolabeled monomeric uromodulin in solution and found that it bound to neutrophils at 4 °C and that the binding could be displaced by sialic acid. Their data with monomeric uromodulin is different from our results with polymeric uromodulin and is consistent with the possibility that monomeric uromodulin involves an interaction with L-selectin, and that monomeric and polymeric uromodulin bind to neutrophils by different mechanisms. In the mammalian kidney, uromodulin exists as a high molecular weight polymer on surface of cells lining the TAL and in the tubular fluid downstream from the TAL. The present study has examined the mechanism of interaction between polymeric uromodulin and neutrophils, and perhaps represents the *in- vivo* situation more closely. Taken together, these other reports and ours do suggest that uromodulin has the potential to participate in the pathogenesis of immune-mediated tubulointestitial nephritis especially in the early stages when neutrophils are an important component of the infiltrating cells.

Furthermore, these results may be significant in the context of neutrophil migration across renal epithelium. Normal urine contains up to 2 million neutrophils per day (25) and an increased excretion of neutrophils in urine (pyuria) occurs in infectious pyelonephritis (26) and in immune-mediated, sterile tubulo-interstitial nephritis (27). While neutrophil migration across the vascular

endothelium has been the subject of many studies (28), neutrophil migration across epithelium in general (29) and renal epithelium in particular (30) has not been thoroughly investigated. Neutrophil migration across the intestinal epithelium has been shown to be mediated by binding of leukocyte integrin, CD11b/CD18 (Mol/Mac-1) to an unidentified ligand in the intestinal epithelium (31). Similar studies have not been performed for renal epithelium. Our data suggest that uromodulin may serve as an integrin ligand facilitating neutrophil migration across renal epithelium in the genesis of pyuria.

In conclusion, we demonstrate that polymeric uromodulin binds neutrophils in a calcium dependent, RGD-mediated, integrin-like process that may be important both in the normal kidney and in inflammatory disorders of the renal interstitium.

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REFERENCES

1. Kumar, S., and Muchmore, A. (1989) Kidney Int. 37:1395-1401.
2. Pennica, D., Kohr, W., Kuang, W.-J., Glaister, D., Aggarwal, B.B., Chen, E.Y., and Goeddel, D.V. (1987) Science 236:83-88.
3. Hession, C., Decker, J.M., Sherblom, A., Kumar, S., Uye, C., Mattaliano, R., Tizard R., Kawashima, E., Schmeissner, U., Heletky, S., Chow, P., Burne, C., Shaw, A., and Muchmore, A.W. (1987) Science 237:1479-1484.
4. Fukuoka, S.-I., Freedman, S.D., Yu, H., Sukhatme, V.P., and Scheele, G.A. (1992) Proc. Natl. Acad. Sci. (USA) 89:1189-1193.
5. Cotran, R.A., and Galvaneck, E. (1979) Contr. Nephrol. 16:126-131.
6. Mayrer, A.R., Kashgarian, M., Ruddle, N.H., Manier, R., Hodson, C.J., Richards, F.F., and Andriole, V.T. (1982) J. Immunol. 128:2634-2642.
7. Hoyer, J.R. (1980) Kidney Int. 17:284-292.
8. Nagai, T. and Nagai, T. (1987) Nephron 47:137-140.
9. Horton, J.K., Davies, M., Topley, N., Thomas, D., and Williams, J.D. (1990) Kidney Int. 37:717-726.
10. Yu, C.-L., Lin, W.-M., Liao, T.-S., Tsai, C.-Y., Sun, K.-H., and Chen, K.-H. (1992) Immunopharmacology 23:181-190.
11. Thomas, D.B.L., Davies, M., and Williams, J.D. (1993) Amer. J. Pathol. 142:249-260.
12. Yu, C.-L., Tsai, C.-Y., Lin, W.-M., Liao, T.-S., Chen, H.-L., Sun, K.-H., and Chen, K-H. (1993) Immunopharmacology 26:249-258.
13. Thomas, D.B.L., Davies, M., Peters, J.R., and Williams, J.D. (1993) Kidney Int. 44:423-429.
14. Kumar, S., Jasani, B., Hunt, J.S., Moffat, D.B., and Asscher, A.W. (1985) Histochem. J. 17:1251-1258.
15. Tamm, I., and Horsfall, F.L. (1950) Proc. Soc. Exp. Biol. Med. 74:108-114.
16. Rhodes, D.C.J., Hinsman, E.J., and Rhodes, J.A. (1993) Kidney Int. 44:1014-1021.
17. Geng, J.-G., Bevilacqua, M.P., Moore, K.L., McIntyre, T.M., Prescott, S.M., Kim, J.M., Bliss, G.A., Zimmerman, T.A., and McEver, R.P. (1990) Nature 343:757-760.
18. Chang, C.D., and Meienhofer, J. (1978) Int. J. Pept. Protein Res. 11:246-249.
19. Diamond, M.S., Staunton, D.E., deForgerolles, A.R., Stacker, S.A., Aguilar, J.G., Hibbs, M.L., and Springer, T.A. (1991) J. Cell. Biol. 111:3129-3139.
20. Ruoslahti, E. (1991) J. Clin. Invest. 87:1-5.
21. Rosen, S.D., Singer, M.S., Yednock, T.A., and Stoolman, L.M. (1985) Science 228:1005-1007.

22. Kirchhofer, D., Grzesiak, J., and Pierschbacher, M.D. (1991) J. Biol. Chem. 266:4471-4477.
23. Gartner, T.K., and Bennet, J.S. (1985) J. Biol. Chem. 260:11891-11894.
24. Tanabe, J., Fujita, H., Iwamatsu, A., Mohri, H., and Ohkubo, T. (1993) J. Biol. Chem. 268:27143-27147.
25. Kesson, A.M., Talbott, J.M., and Gyory, A.Z. (1978) Lancet 2:809-812.
26. Stamm, W.E. (1983) Amer. J. Med. 75:53-58.
27. Cameron, J.S. (1988) Quart. J. Med. 66:97-115.
28. Springer, T.A. (1994) Cell 76:301-314.
29. Parsons, P.E., Sigahara, D., Cott, G.R., Mason, R.J., and Henson, P.M. (1987) Amer. J. Pathol. 129:302-312.
30. Cramer, E.B., Milks, L.C., Brontoli, M.J., Ojakian, G.K., Wright, S.D., and Showell, J.J. (1986) J. Cell. Biol. 102:1868-1877.
31. Parkos, C.A., Delp, C., Arnaout, M.A., and Madara, J.L. (1991) J. Clin. Invest. 88:1605-1612.