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Measurement of the kinetics of protein uptake by proximal tubular cells using an optical biosensor

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Background. The affinity and specificity of protein reabsorption by proximal tubular cells have been investigated using techniques for monitoring endocytosis, demonstrating a high capacity but low affinity process. It is not known whether uptake is through binding to a single binding site/receptor with differing affinities, or if there are several classes of binding sites receptors, each specific for differing proteins or groups, such as, high or low molecular weight proteins.

Methods. We have developed a novel technique for analyzing the kinetics of protein binding to tubular cells using a optical biosensor system. We have studied the binding of cultured LLCPK cells to albumin and RBP immobilized onto the sensor. By adding increasing concentrations of competing proteins [varying in molecular weight from 66,000 to 11,800 D and pI from 4.6 to 9.2 as represented by albumin, alpha1-microglobulin (α_1M), retinol binding protein (RBP), cystatin C and beta2-microglobulin (β_2m)], specific and inhibitable cell binding was demonstrated.

Results. Equilibrium constants, K_A, could be calculated from the reciprocal of the protein concentration causing 50% inhibition in binding rate. These were: albumin = 8.0×10^4 M^{-1}, α_1M = 2.0×10^5 M^{-1}, RBP = 2.7×10^4 M^{-1}, cystatin C = 2.0×10^4 M^{-1}, β_2m = 4.2×10^3 M^{-1}. There were no significant differences between the measured K_A's whether RBP or albumin were immobilized on the surface.

Conclusions. All the proteins gave similar shaped inhibition profiles, suggesting that there is one binding site/receptor for all proteins studied, regardless of molecular weight or charge, but there are differing affinities for each protein.

Our current understanding of the process of reabsorption of proteins from the glomerular filtrate, by the proximal tubule, stems from the pioneering experiments of Maunsbach in 1966 [1, 2]. Proteins in the luminal space are exposed to the dense brush border of the proximal tubular epithelial cells. Proteins then travel to the apical plasma membrane reabsorptive pits, and it is possible that the geometry of the brush border introduces some steric hindrance to the passage of the proteins, acting as a size selective filter.

It is now generally accepted that proteins are mainly taken up from the tubular lumen by an adsorptive endocytic process (which is generally receptor mediated) with only a small proportion taken up by fluid phase endocytosis (non-receptor mediated) [3, 4]. The final step of protein reabsorption is the binding of a protein molecule to a site or possibly a binding site or receptor on the proximal tubular cell, followed by localization into clathrin coated invaginations that form vesicles for subsequent internalization [4 -7]. There is evidence to suggest that internalization is triggered by binding to protein complexes called adapter proteins, which together with clathrin form the components of the coating on the plasma membrane that enclose the membrane vesicle [8, 9]. Proteins also bind to sites on the microvilli, but it is not clear whether lateral migration occurs into the endocytic invaginations [10]. Following internalization and prior to fusing with lysosomes, the proteins detach from the membrane binding sites and are then transferred to the lysosomes and degraded into amino acids [11, 12]. The binding sites are then recycled back to the apical plasma membrane [13].

The initial binding process is viewed as an interaction between the positively charged groups on the proteins and the negatively charged sites on the apical cell membrane, thus facilitating the absorption of cationic proteins [10]. However, anionic proteins are also efficiently reabsorbed, and no simple relationship between the number and density of net positive charges and the selectivity of protein absorption has been shown [14]. This model suggests that there will be no competition for reabsorption between proteins of the same charge. Alternatively, it has been proposed that whatever their size and charge, different proteins compete with each other for the uptake process. The proteins bind

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to a common binding site or receptor with different affinities according to their molecular characteristics [15–18].

Several studies have shown that the reabsorption of protein process is a high capacity, low apparent affinity and a saturable process [3, 10, 14, 19–22]. They have provided evidence that protein uptake is via a mechanism that has some of the characteristics of a receptor mediated process, but there are only a limited amount of quantitative data on the kinetics and specificity of renal tubular uptake for different proteins. More recently, using *in vitro* methodologies, there is growing evidence for competition between different proteins [14]. Simonnet et al have shown that there is competition between β2-microglobulin (β_2m), albumin and other low molecular weight proteins [20]. There is some evidence for two binding sites for β_2m and albumin, with high and low affinity binding sites reported [20, 22], but their existence has not been confirmed in any other study using different experimental conditions [4].

When binding interactions that trigger endocytosis are characterized, it is important to differentiate the faster kinetics of the initial binding event from the slower and irreversible internalization process. This has been achieved by the use of inhibitors of the internalization process, such as cytochalasin B or by the use of low temperatures to reduce membrane fluidity. The methods used for assessing the kinetics and specificity of protein binding to proximal tubular cell membranes have been many and varied, but have until recently all used either radio- or fluorescent-labeled proteins with microscopy or saturation analysis techniques [4, 10, 13, 20, 22]. These approaches are likely to overestimate the affinity of binding, especially of such a low affinity process as the proximal tubular uptake of proteins. The affinity of binding is also temperature dependent and the use of reduced temperatures would also introduce the same bias. Internalization can also be prevented by the use of brush border membrane fragments, but the preparation of this material may damage the surface binding sites.

We have set out to measure the kinetics of the initial binding event of endocytosis using an optical biosensor. This technology has several advantages over the more conventional technologies mentioned above [23]. Continuous real-time monitoring, without the use of labeled material, should enable the kinetics of protein binding to proximal tubular cells to be measured at near physiological temperatures and without the use of inhibitors of endocytosis. The IAsys™ instrument (Affinity Sensors Ltd, Cambridge, UK), which is based on a resonant mirror mounted in a stirred cuvette, monitors the interactions between pairs of molecules, where one partner is immobilized on the sensor surface and the other is in solution.

The aims of our study were to determine the affinity of binding of a range of proteins with differing sizes and charges to a porcine proximal tubular epithelial cell line (LLCPK), and to investigate whether there is evidence for a common binding site or receptor for all proteins.

METHODS

Materials

All purified human proteins (>95% purity) were a kind gift from SCIPAC Ltd. (Sittingbourne, Kent, UK) apart from purified human serum albumin (DadeBehring Incorporated, Marburg, Germany) and cystatin C (a gift from Prof. A. Grubb, Lund, Sweden). Rabbit anti-human polyclonal antibodies for β_2m, retinol binding protein (RBP), and α1-microglobulin (α_1M) were purchased from Dako Ltd (High Wycombe, Bucks, UK). A monoclonal antibody raised against human serum albumin was a gift from DadeBehring Incorporated (Wilmington, DE, USA). LLCPK cells were obtained from the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology & Research (Porton Down, Salisbury, UK). All cell culture plastics were from Falcon Labware (Oxford, UK). All other chemicals were obtained from Sigma (Poole, Dorset, UK).

The optical biosensor system

The instrument performs real-time monitoring of bimolecular interactions between an immobilized ligand and its binding partner producing a plot of response, measured in arc seconds, against time [23]. The open stirred cuvette format of IAsys™ has the advantage of allowing an analysis between particulate suspensions such as cell-protein interactions to be monitored with minimal complications of aggregation, fouling and subsequent blockage of the instrument. The mathematical theory describing kinetic rate analysis follows the Langmurian assumption of a simple bimolecular interaction to a single binding site, with all the binding sites kinetically homogeneous and binding independently of each other. The measurement of association rates for cell-protein interactions is much more complex as it involves multiple interactions of low affinity binding involving multiple binding sites (there can be as many as 10^5 to 10^6 receptors per cell). The dissociation reaction might be also be slow or negligible due to multiple interactions and rebinding of the complex [24–26]. Because of this complexity, kinetic rate analysis cannot easily be used to determine affinity constants for cellular interactions. One of the alternative approaches to measure affinity constants when avidity or rebinding is a problem is to measure the degree of inhibition of binding in the presence of a range of free ligand concentrations. This method also allows the measurement of affinity constants for different competing ligands over a wide range of binding constants [24, 25]. The association phase is described by the equation:

$$R_t = R_0 + E[1 - \exp(^{-k}on\ ^{t})]$$

where R_t is the response at time t, R_0 is the initial response, E is the extent of the change in response and k_{on} is the

Table 1. The immobilization conditions used for different proteins and the resultant cuvette surface concentrations.

Protein	Mr *Da*	pI	10 mM formate buffer *pH*	Concentration[a] *μg/ml*	Amount immobilized on cuvette surface *pmoles*
β_2m	11,800	5.3	4.5	5.0	2.20
RBP	21,000	5.6	4.5	8.9	0.80
α_1M	32,000	5.5	4.5	13.6	2.25
Albumin	66,000	4.6	3.5	28.0	1.5

This table contains representative data from a single immobilization. Abbreviations are: β_2m, β2-microglobulin; RBP, retinol binding protein; α_1M, alpha1-microglobulin.

[a] Concentrations all equivalent to 85.0 pmoles of protein added to the cuvette

on-rate constant. This equation can be fitted to the association part of the binding interaction using a single exponential model mathematically to derive k_{on}. Alternatively, the initial rate of binding can be used in place of k_{on}, calculated by linear regression from the initial linear portion of the association curve, after baseline correction [23]. The initial rates or k_{on} determined for the different inhibitor concentrations are expressed as a percentage of the uninhibited initial rate or k_{on} and plotted against the inhibitor concentration [24]. The effective concentrations of free inhibitor causing a 50% inhibition of binding rate, the IC_{50}, are calculated and the equilibrium/affinity constant K_A derived by applying the relationship:

$$K_A = 1/IC_{50}\ M^{-1}$$

Protein immobilization

Proteins were immobilized on the carboxy methyl dextran (CMD) IAsys™ cuvette (Affinity Sensors) using an EDC/NHS chemistry [Amine coupling kit containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxy-succinimide (NHS), and 1 M ethanolamine, pH 8.5; Affinity Sensors]. The dextran surface was activated by adding equal volumes of 0.4 M EDC and 0.1 M NHS solution in deionized water for eight minutes, then replacing it with PBS-Tween [10 mM sodium phosphate, pH 7.4, 138 mM NaCl. 2.7 mM KCl, 0.05% (vol/vol) Tween 20 (Surfact-Amps 20™; Pierce & Warriner)], for five minutes to establish a pre-immobilization baseline response. Proteins dissolved in the appropriate coupling buffer were immobilized for 10 minutes at equimolar concentrations to achieve comparable surface densities. A list of proteins immobilized and the pH and concentration conditions are given in Table 1 (0.01 M formate buffer at pH 3.5 for albumin and at pH 4.5 for the other proteins). Residual NHS esters were quenched by adding 1 M ethanolamine pH 8.5 for two minutes. A final PBS-Tween wash was carried out to establish a post-immobilization baseline response on the biosensor. The surface was regenerated with 20 mM HCl for one minute then returned to PBS-Tween. The difference in the pre- and post-immobilization responses was used to calculate the amount of protein immobilized on the surface by applying the relationship that 1 ng protein/mm^2 gives a response of 163 arc secs [23, 27]. The biological activity of the immobilized protein was confirmed by binding to their respective specific antibodies diluted in PBS-Tween; (β_2m, 1:1000; RBP, 1:100; α_1M, 1:100; and albumin, 1:10) and the binding response measured. This interaction between the protein and its specific antibody was used, at regular intervals throughout the experiment, to assess and monitor stability and performance of the cuvette surface. All antibody and cell-protein interactions were studied on the IAsys™ at 25°C.

Cell culture

LLCPK cells [27, 28] were grown to confluence in 80 mm^3 plastic culture flasks in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal calf serum (Sigma Chemical Co.) in a 37°C incubator (IR 1500 Automatic CO_2 Incubator; Flow Laboratories) with a humidified atmosphere of 95% air and 5% CO_2. For uptake experiments, confluent cells were trypsinized in 0.25 g/liter trypsin (Sigma) made up in Ca^{2+} and Mg^{2+} free EDTA containing phosphate buffer (0.02% wt/vol) for 10 minutes, and after centrifugation the cells were resuspended in a serum free medium, passed through a 70 mm cell strainer (Falcon Labware) and then gently through a 22 gauge needle to achieve a monodispersed cell suspension. The presence of a brush border on the trypsinized cells was confirmed by electron microscopy as shown in Figure 1. Cell viability was assessed by using the trypan blue dye exclusion technique, counting before and after each interaction analysis. All binding experiments were performed using cells between passages 104 and 110.

Experimental procedures

All cell binding experiments were performed using signal responses recorded at two second intervals. Firstly, 100 μl of PBS-Tween was added and allowed to equilibrate and the baseline to stabilize, then 100 μl of cell suspension was added and the binding interaction monitored for three minutes. After this the cells were aspirated and 200 μl of PBS-Tween added for two minutes to assess dissociation. The surface was regenerated with 20 mM HCl for one minute, then returned to PBS-Tween and a baseline re-established, and the difference between the pre- and post-interaction baselines was used to correct for baseline drift.

Mass transport effects. The amount of reactant in the bulk solution delivered to the immobilized ligand on the sensor surface, that is, the cells to the immobilized protein, depends on the rate of diffusion and the concentration gradient across the boundary layer in contact with the immobilized ligand. In order to achieve binding rates with minimal influence from mass transport, three different stir speeds were tested (15, 30 and 45 rpm), and the potential

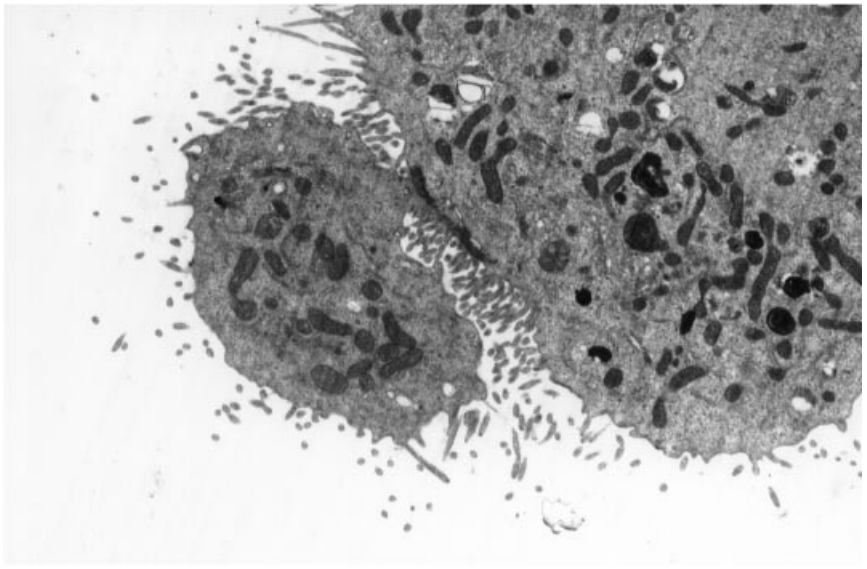

Fig. 1. Electron micrograph of trypsinized LLCPK cell showing residual brush border structures.

influence of mass transfer was examined by monitoring binding of 50,000 cells/cuvette in the presence (5 mg/ml) and absence of free human serum albumin. The serum albumin was added to mimic the viscosity effects that would be introduced during the inhibition experiments, which could cause additional mass transport effects [29].

Cell number versus response. To determine whether binding signal corresponded to the increasing cell number and to establish the appropriate cell number for the inhibition experiments (a binding profile for a range of cell numbers, 25 to 75,000 cells per cuvette, to each of the four immobilized protein surfaces indicated in Table 1), each reaction was monitored in triplicate.

Specificity of binding. The specificity of binding of the four immobilized protein surfaces to cells was investigated by demonstrating displacement of binding with increasing concentrations of their respective free protein in solution.

Nonspecific binding. Careful consideration of controls was required to establish whether specific cell:protein binding was occurring with minimal nonspecific interactions. This was explored in three ways using the RBP immobilized surface by monitoring binding with: (*i*) 200 μl of serum free medium alone, (*ii*) 50,000 cells/cuvette in the presence of 80 pmoles of RBP (a 100-fold molar excess over the immobilized protein), and (*iii*) a range of concentrations of each of the free proteins to be used in the inhibition study.

Measurement of inhibition profiles on RBP and albumin immobilized sensor surfaces. Inhibition profiles were generated on the two surfaces from five different concentrations (equimolar concentrations ranging from 0 to 64 nmoles) of each of the five proteins prepared in PBS-Tween: β_2m, cystatin C, RBP, α_1M and albumin. After preincubation with the free protein (100 μl) in the cuvette, 100 μl of 5×10^5 cells/ml (50,000 cells/cuvette) were added and binding monitored. Measurements of each inhibition profile were performed in duplicate.

Statistical analysis

The initial rate calculated by linear regression analysis was assessed for goodness of fit and accepted when $r = 1.00 \pm 0.02$. All data are represented as the mean $\pm$ SEM for each analysis. Comparison of the K_A values on the two sensor surfaces was made using paired *t*-test analysis using Statview® (Abacus Concepts, CA, USA), on an Apple Macintosh computer.

RESULTS

Increasing the stir rate from 15 to 45 rpm moderately increased the rate of binding, but the greatest change occurred between 15 and 30 rpm. A stir speed of 40 rpm was chosen for all subsequent experiments. Cell viability, assessed at the end of each analysis by counting viable cells using trypan blue exclusion technique, was $> 95\%$, confirming that no significant damage had occurred to the cells during each binding experiment.

Initial rates were calculated from the binding curves obtained on the four equimolar immobilized protein surfaces with different cell densities. These were plotted against the cell number, as demonstrated in Figure 2. An increasing binding response with increasing cell number was observed, reaching a plateau at 75,000 cells, on all surfaces except for β_2m. The rate of binding was lower as the size of the protein immobilized on the surface increased: albumin $<$ α_1M $<$ RBP $<$ β_2m. The binding rate for β_2m was higher and still increasing linearly at the highest cell number. From these results, 50,000 cells/cuvette was chosen for all subsequent experiments.

Specificity of binding to the four immobilized proteins was demonstrated by showing inhibition of binding by

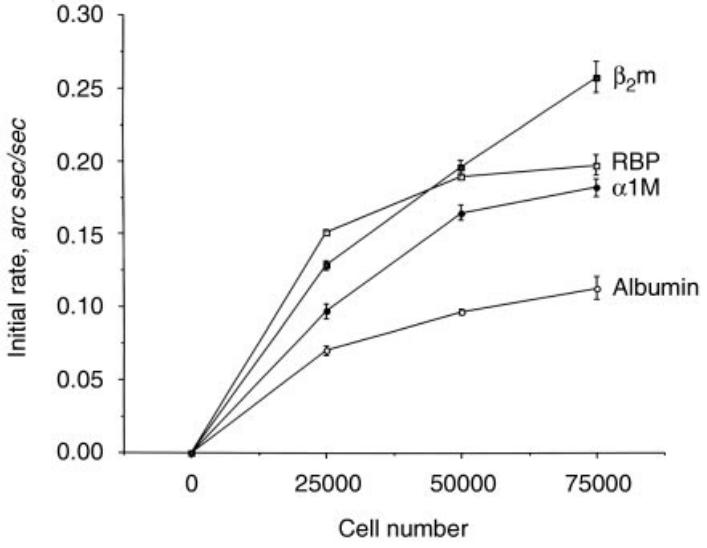

Fig. 2. Graph showing increasing cell binding rates with increasing cell number on the four proteins immobilized on the sensor surface but at different rates.

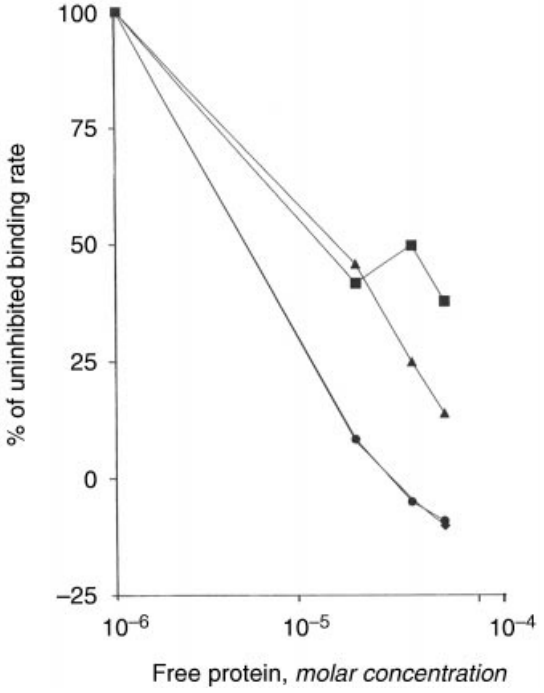

Fig. 3. Graph showing specific inhibition of binding of 50,000 LLCPK cells to proteins immobilized on the surface with their respective free proteins. Symbols are: (■) β2-microglobulin; (▲) retinol binding protein; (●) α1-microglobulin; (♦) albumin. Note that α1-microglobulin and albumin are superimposed until 10^{-4} concentration.

adding increasing concentrations of their respective free protein. The results were expressed as percentage binding and plotted against the free protein concentrations. A decrease in binding with increasing free proteins was demonstrated for all four surfaces (Fig. 3). Complete inhibition of binding was demonstrated on both the α_1M and albumin surfaces with the addition of their respective free proteins. RBP and albumin surfaces were chosen to represent the low and the high molecular weight protein surfaces respectively, to establish affinity constants for each of the five proteins.

There was neither nonspecific-binding of serum free medium nor the soluble proteins to the RBP surface. The excess free RBP completely inhibited cell binding (Fig. 4). Furthermore, as shown in Figure 3, both free albumin and α_1M also completely inhibited cell binding, confirming the lack of any significant nonspecific cell binding to either of the protein surfaces.

Using the RBP and albumin immobilized surfaces, the association phase of the binding curve followed for three minutes produced an absolute response of about 30 to 40 arc seconds. Initial rates and k_{on} were calculated from the first 100 seconds of data; however, since the k_{on} curve fits were poor the initial rate data were used thereafter. Initial rates calculated from the binding curves were expressed as % binding of the uninhibited rate and plotted against the molar concentration of free proteins, as demonstrated in Figure 5. All five proteins showed a decrease in binding with increasing amounts of soluble protein in the cuvette on both the surfaces. The amount of free protein required to achieve 50% of inhibition of binding was different for each of the proteins; however, the inhibition profiles for each protein on the two surfaces were very similar in shape, as depicted in Figure 5 A and B.

The affinity constants calculated from these inhibition profiles are shown in Table 2. α1-Microglobulin showed the highest value 3.07×10^5 M^{-1}, which was 50-fold greater an affinity than β_2m, tenfold higher than RBP and about threefold difference with albumin. The affinity constant values were very similar for cystatin C and RBP, while that for β_2m was the lowest at 4.35×10^3 M^{-1}. When K_A values for the different proteins on the two sensor surfaces were compared, using the paired *t*-test analysis, no significant difference was demonstrated between the two. However, statistical analysis of the combined data from the two surfaces showed that significantly different K_A values were obtained between each protein ($P < 0.05$), except for RBP and cystatin C.

DISCUSSION

The use of cultured proximal tubular epithelial cells (PTEC) to study protein reabsorption is well established [22, 27]. PTEC such as the LLCPK line are polarized and grow as an adherent cell line in culture with the brush border towards the media [28]. LLCPK cells retain a defined brush border even after the mild trypsin treatment used to remove them from the culture vessels, and they have been well characterized as possessing many of the essential functional characteristics of a PTEC including the

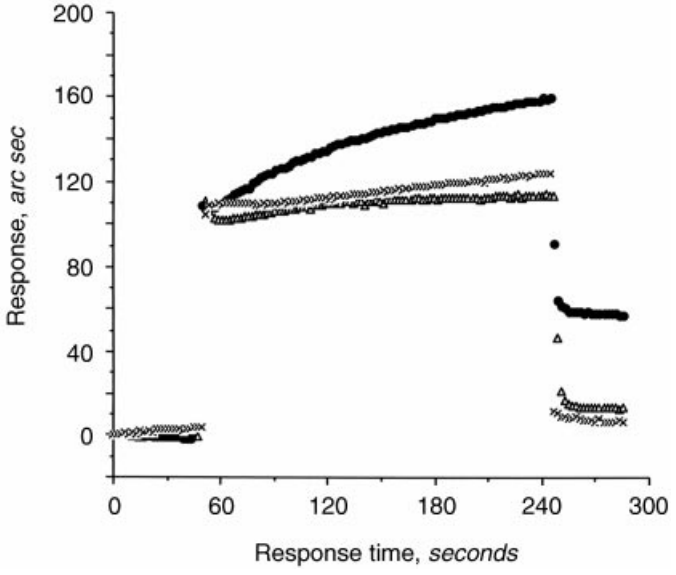

Fig. 4. An overlay graph showing specific binding of 50,000 LLCPK cells (●) to RBP immobilized on the sensor surface and nonspecific binding with excess free retinol binding protein (x) and with serum free medium alone (△). An initial bulk refractive index shift was observed as the cell suspension in the medium was added. This signal change was subtracted from the binding responses during the background correction procedure. This was followed by a binding or association phase. The addition of PBS-Tween caused another bulk shift response followed by the very limited dissociation phase. In this model of cell-protein interactions, no significant amount of dissociation was achieved since rebinding of the complex occurred due to the multivalent nature of cells.

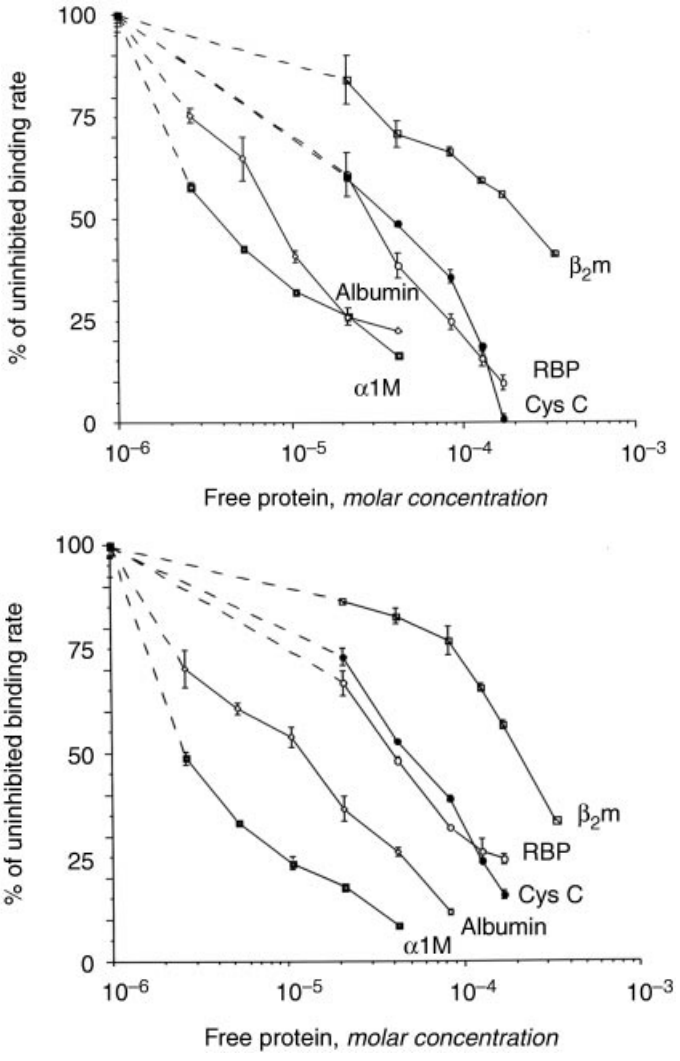

Fig. 5. (*A*) Inhibition of binding profile on an albumin immobilized sensor surface and (*B*) inhibition of binding profile on an RBP surface with equimolar concentration of free proteins in the cuvette.

protein reabsorption characteristics [27]. While other groups have used PTEC as adherent monolayers because this approach retains cell orientation, we have used the cells in free solution so they would be randomly oriented. However, as the brush border is retained to a certain extent (Fig. 1) and as this is the region where the vast majority of the protein binding can be expected to occur, binding to the sensor surface will predominantly, if not exclusively, reflect brush border interactions. While cellular projections such as brush border may facilitate binding to the sensor surface, the cells appear to remain intact during the experiment, and there is no apparent shedding of cellular extrusions. The cuvette design of the IAsys™ enables cells to be recovered following an experiment and cell viability was found to remain at >95%, suggesting that minimal damage had occurred as a result of the mixing process.

Optical biosensors such as the IAsys™ respond to changes in mass occurring at the sensor surface, that is, the higher the mass the greater the response [23]. The evanescent field, propagating away from the sensor surface, only probes the first 200 to 300 nm, which is very small in comparison with the cell diameter of approximately 10 μm, and so only a small portion of the cell will enter the evanescent field. The depth of the dextran layer can be affected by pH and ionic strength of the medium and by proteins cross-linking the chains [23]. The conditions used in these experiments provided suitable conditions for monitoring LLCPK cellular interactions, but different conditions may be required for different cell types.

Nonspecific adhesion to surfaces is a significant problem and it was therefore necessary to apply careful controls to ascertain that specific cell-protein interactions were being monitored by the biosensor. In a classical receptor-ligand binding model, nonspecific or nonsaturable binding is measured by adding a large excess concentration of the free protein, and if this displaces all the bound protein from the receptor then there is minimal nonspecific binding [30]. This was demonstrated in our study by RBP, albumin and α_1M in separate experiments; all completely inhibited the binding of the LLCPK cells, indicating that specific interactions between immobilized proteins and cells were monitored.

Table 2. Summary of measured equilibrium constants (K_A) on each protein surface with a summary of the molecular weight and charge characteristics of the different proteins

	K_A on albumin (M^{-1}) ± SEM	K_A on RBP (M^{-1}) ± SEM	Mean K_A (M^{-1}) ± SEM
β_2M	$4.14 \times 10^3 \pm 7.5 \times 10^1$	$4.57 \times 10^3 \pm 1.3 \times 10^2$	$4.35 \times 10^3 \pm 1.4 \times 10^2$
CystatinC	$2.54 \times 10^4 \pm 9.5 \times 10^2$	$1.96 \times 10^4 \pm 2.0 \times 10^2$	$2.25 \times 10^4 \pm 1.7 \times 10^3$
RBP	$3.28 \times 10^4 \pm 4.2 \times 10^3$	$2.57 \times 10^4 \pm 6.5 \times 10^2$	$2.92 \times 10^4 \pm 2.7 \times 10^3$
α1M	$2.36 \times 10^5 \pm 1.4 \times 10^4$	$3.78 \times 10^5 \pm 7.5 \times 10^3$	$3.07 \times 10^5 \pm 4.1 \times 10^4$
Albumin	$1.20 \times 10^5 \pm 1.5 \times 10^3$	$7.62 \times 10^4 \pm 1.3 \times 10^4$	$9.78 \times 10^4 \pm 1.3 \times 10^4$

P value = 0.45 between the two surfaces; No significant differences. P value < 0.05 between proteins, that is significantly different values for each protein. Abbreviations are in Table 1 legend.

The binding response of LLCPK cells to the four immobilized protein surfaces showed rates of binding inversely proportional to the size of the immobilized proteins. This suggests that there may be a steric hindrance to the cell binding to the dextran-immobilized protein. The binding of cells to the larger sized immobilized protein within the crowded matrix may cause a restrictive accessibility to the binding site/receptor on the cell [23]. The binding sites/receptors on the cell surface may be inaccessible, however, the flexibility of the linear dextran chains (200 to 500 nm in depth) on the sensor surface would enable the brush border structures to penetrate to a significant extent. The need to "penetrate" the dextran layer for specific binding to occur can introduce sufficient steric hindrance to modify the measured rate constant, as two processes would now be involved (penetration and binding) [23]. However, this first stage of the interaction should be reasonably consistent for all the proteins studied as the molar amounts immobilized were kept as similar as possible.

For measuring affinity constants two protein surfaces were chosen, RBP and albumin, for immobilization on the sensor surface representing the low and high molecular weight proteins. β2-microglobulin was not chosen because a different shaped binding curve (Fig. 2) was obtained, possibly due to the presence of a second known receptor (Class I antigen) on the cell surface, which could complicate the interpretation of the inhibition curves [31]. Indeed, exchange of cell surface β_2m can occur readily, as we have shown in previous studies [26, 32]. α1-Microglobulin was also not chosen for the inhibition experiments as it is an intermediate size protein between RBP and albumin. Cystatin C was also not immobilized on the surface, since as a basic protein with a pI of 9.2, immobilization would require an alternative indirect conjugation procedure such as streptavidin or a biotin/avidin system.

Our experiments showed that the low molecular weight (LMW) proteins were, in general, less inhibitory than the larger molecules on both of the surfaces. This suggests that binding affinity was proportional to molecular weight or that the number of possible binding/receptor sites were more for these molecules. However, this is not in accord with α_1M having a higher affinity of binding than albumin. The range of pI's for the proteins was 4.5 to 9.2 and there was no apparent correlation between protein pI and measured K_A, showing that protein:PTEC binding is not simply a charge-mediated electrostatic interaction.

Binding constants cannot be easily derived from the association (on-rates) and dissociation (off rates) curves generated experimentally for a multivalent complex system [23–25]. The measurement of association rates for cell-protein interactions, with multiple binding sites, would be quite complex to model mathematically and so would the measurement of their dissociation rates, since the dissociation would be slow or negligible, as indeed was observed in our studies (Fig. 4). Nieba, Krebber and Pluckthun, using another optical biosensor, the BIAcore™ (BIACORE, Stevenage, UK), showed that the inhibition approach could be used for the determination of mono- and multivalent binding constants and that these compared well, at least in proportion, with those obtained from free solution binding [24]. The other advantage of this competitive kinetic method was that direct comparison of affinities of different proteins was possible using the same sensor surface. The assumption underlying this method is that the initial rate constant describing the observed association phase is proportional to the on-rate (k_{on}), this being valid when the k_{diss} (dissociation rate constant) is negligible, as found in our study.

In this study displacement curve analysis was applied to estimate the relative affinities of the free proteins. Occasionally, in such experiments, a curve may display a stepped or terraced appearance, suggesting competition at more than one class of receptor sites [33]. The inhibition profiles showed monophasic curves for all the proteins, except possibly for β_2m. These steps in the curve may also overlap, giving rise to a gradual displacement, which in our experiment seems to be the case with β_2m. If the uptake mechanism were different for different classes of protein such as the LMW and the high molecular weight (HMW) proteins, then different inhibition profiles would have been observed for the proteins on the two immobilized surfaces. The shape of the inhibition profiles of the LLCPK cells was the same regardless of the protein immobilized on the surface, and statistical analysis showed no significant difference in the affinity constants calculated. We concluded from these findings that there was no evidence for two

binding sites, but a strong implication that there was a common receptor or binding site for all the proteins studied.

The proximal tubular cells showed different rates of binding to each protein and overall the affinities measured were lower (10^3 to 10^5 M^{-1}) compared to values obtained from previous studies (Table 2). Simonnet et al measured the kinetics of labeled β_2m, albumin and other low molecular weight proteins on isolated brush border membrane preparations at 37°C, and reported two affinities for β_2m: 4.0×10^6 and 1.0×10^5 [20]. The binding of the T-cell receptor to the class I HLA:peptide:β_2m heterotrimer has an apparent K_A of approximately 1.54×10^5 M^{-1} [34, 35], which is similar to our measured values. The definition of biologically significant thus can encompass the kind of affinity constants we have recorded for protein interactions with proximal tubular epithelial cells.

Brunskill et al demonstrated that binding of ^{125}I-albumin to opossum (OK) cells at 4°C had reached equilibrium after 24 hours [22]. Schwegler et al used FITC-albumin and OK cells at 37°C with a 15 minute incubation [4], and Simonnet et al separated the bound fraction of ^{125}I-β_2m to isolated brush border preparations from the free fractions after one minute [20]. These time points suggest that equilibrium will not have been achieved in all of these studies. The low temperature conditions would enhance the affinity of all interactions, especially the low affinity interactions, and this might explain the second lower affinity binding site of Brunskill, Nahorski and Walls [22].

Several investigators have identified possible albumin receptors in endothelial cells as potential glycoprotein scavenger receptors mediating binding and subsequent endocytosis of modified albumins [36, 37]. Brunskill et al also suggested similar potential scavenger receptors for albumin reabsorption in kidney proximal tubules [22]. Recently, Christensen suggested that megalin/gp330 might be a scavenger receptor for reabsorption of serum albumin [38]. If these scavenger receptors are being identified for albumin, then it is perhaps feasible to suppose that these receptors may also mediate the uptake of other proteins. In nature, small chaperone molecules have been identified as scavenger receptors functioning characteristically as one receptor or binding site with different affinities for a range of ligands [39].

Further work is required to identify the putative common binding site/receptor, and it would be essential to confirm our findings using primary proximal tubular cells. We conclude that our data are consistent with the existence of a common binding site/receptor for protein reabsorption in cultured proximal tubular epithelial cells regardless of size or charge. The steric hindrance or geometric constraint of the native brush border may be the rate limiting process to the overall rate of the epithelial cell endocytic processes, but the affinity of the ultimate binding interaction would still be an important independent parameter to discern. The importance of our work, we believe, is that it enables a wide variety of proteins to be studied in one series of experiments, indeed more than in any other published study.

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APPENDIX

Abbreviations used in this article are: α_1M, α1-microglobulin; β_2m, β2-microglobulin; DMEM, Dulbecco's modified Eagle's medium; HMW, high molecular weight; K_A, equilibrium constant; k_{diss}, dissociation rate constant; k_{on}, on rate; LLCPK, porcine proximal tubular epithelial cell line; LMW, low molecular weight; PTEC, proximal tubular epithelial cells; RBP, retinol binding protein.

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