BINDING AND UPTAKE OF ALBUMIN BY OPOSSUM KIDNEY CELLS

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by

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ABSTRACT

The presence of protein in the urine of patients with renal disease is an adverse prognostic feature, such that those patients with proteinuria are more likely to develop progressive renal failure than those without proteinuria. The hallmark of progressive renal disease is the development of renal interstitial fibrosis and scarring, and even in the primary glomerular diseases the progression of renal disease correlates most closely with the tubulo-interstitial pathology. This association of proteinuria with tubulo-interstitial scarring, and progressive renal failure has led to the hypothesis that filtered protein, most notably albumin, exerts a toxic effect on the proximal tubular epithelium, damaging these cells and thus initiating the process of interstitial inflammation and subsequent scarring.

If it is postulated that proteinuria may be pathophysiological in disease states, then there may be some utility in trying to block the re-absorption of protein in the proximal tubule. However the mechanism of binding and regulation of uptake of proteins by proximal tubular cells is poorly understood. The aims of the thesis are therefore to clarify the characteristics and specificities of albumin binding to proximal tubule derived opossum kidney cells and to identify potential receptor molecules. In addition, the mechanism of regulation of receptor mediated endocytic uptake of albumin will be examined giving particular attention to the role of heterotrimeric GTP-binding proteins, protein kinase C and phospholipase D.

Albumin binding to opossum kidney cell monolayers has been measured using [^{125}I]-albumin, and affinity constants derived for binding sites using non-linear curve fitting. Lectin competition studies have been performed to further characterise the receptor, and numerous other competitors of binding have been employed in order to classify the receptor. [^{125}I]-albumin ligand blotting and lectin blotting of opossum kidney cell membranes has been used to identify receptor proteins.

Assays of both fluid phase endocytosis and receptor mediated endocytosis have been developed in opossum kidney cells. The regulation of the endocytic pathway has been examined using a number of potential inhibitors. In particular bacterial toxins have been employed to identify potential points of regulation of the pathway by GTP-binding proteins. The apical endocytic pathway in these cells has been examined morphologically using electron microscopy of gold-albumin.

Based on the results of the above experiments a rat cDNA encoding the G-protein subunit Gi3α has been subcloned into pcDNA3. This vector has been stably transfected into opossum kidney cells by the calcium phosphate method. Over-expressing transfects have been selected and screened by western blotting and immunocytochemistry. These stable transfects have been used to measure albumin endocytosis and the results compared to control transfects and wild type cells.

Two binding sites for albumin have been identified, each with a different affinity. Based on the lectin competition studies the receptors appear to be glycoproteins carrying O-linked sugars. Specificity experiments indicate that the receptors share many similar characteristics to the family of scavenger receptors. [^{125}I]-albumin ligand blotting has revealed the presence of three specific albumin binding proteins.

Endocytosis has been visualised using electron microscopy, with gold- albumin being seen in multiple intracellular vesicular structures. These endocytic pathways can be regulated by GTP-binding protein modulating agents. Opossum kidney cells have been successfully transfected with the $G_{i3\alpha}$ protein subunit. These cells show enhanced uptake of albumin compared to controls.

Therefore the experiments described in the thesis document the characteristics of albumin binding to opossum kidney cells, identify the potential receptors involved, and explore the mechanism of regulation of the subsequent endocytic uptake of albumin by the cells.

BINDING AND UPTAKE OF ALBUMIN BY OPOSSUM KIDNEY CELLS

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Chapter 1

INTRODUCTION

1.1 The Incidence and Natural History of Chronic Renal Failure

It has been stated that more than 3200 people develop end-stage renal disease each year in the United Kingdom (Anonymous 1994a). At this stage the affected individuals require treatment by dialysis if they are to survive. The provision of dialysis is expensive, costing the National Health Service approximately £20 000 per patient each year. Dialysis however is a poor substitute for native renal function, patients on dialysis having both a lower quality of life and a shorter life expectancy than equivalent individuals with preserved renal function.

For these reasons it would be desirable, if possible, to prevent the development of chronic renal failure and thus abrogate the need for dialysis. Unfortunately once initiated renal failure tends to be relentlessly progressive. The nature of the initiating insult may be well defined, but even when it subsides or is removed the renal disease in many instances continues to progress. Furthermore, therapeutic strategies designed to slow or stop this progression have so far been largely ineffective.

Renal responses to injury may themselves be in some way accountable for the subsequent gradual loss of remaining renal function. It is clear that kidney glomeruli, tubules and interstitium respond to a very wide variety of insults in a similar fashion, and the kidney's repertoire of

response to injury is strictly limited (Klahr *et al* 1988). This is most apparent when the "end stage" kidney is examined; it is almost always impossible to determine the cause of the initial insult, and it appears that numerous pathogenic stimuli act to provoke sclerosis of renal tissue by a common mechanism, and that once initiated this process is self-perpetuating.

The appearance of the end-stage kidney is characteristic (Heptinstall 1983). Macroscopically, the kidney appears small and shrunken. Microscopically, the glomeruli demonstrate loss of capillary structure with localised areas of cell proliferation, and progressive scarring results in collapse and sclerosis of the capillary bed. Tubular atrophy is marked, especially in those tubules attached to scarred glomeruli, and often the tubules may be surrounded by inflammatory cells. The interstitium also displays prominent fibrosis and scarring with deposition of collagen and lipids. Large numbers of fibroblasts are seen. A similar pattern of evolution of both tubulo-interstitial sclerosis and glomerular sclerosis is observed in both humans and animals with chronic renal failure, and impairment of renal function correlates with these histological changes.

1.2 The Relationship Between Tubulo-interstitial Scarring and Progression of Renal Failure

A consistent finding in patients and animals with renal disease is that declining renal function correlates most strongly with the pathological

tubulo-interstitial changes. This observation holds true even in primary glomerular diseases, and therefore it has been suggested that the key events in the development of progressive renal scarring occur in the kidney tubules and/or interstitium.

Risdon *et al* (1968) found a highly significant correlation between the serum creatinine, creatinine clearance and the extent of tubular damage in the renal biopsies of a group of patients with glomerulonephritis. The correlation between glomerular damage and the measures of renal function was less significant. Notably it was seen that some individuals with severe glomerular damage but little tubular damage had a normal creatinine clearance, whereas some individuals with essentially normal glomeruli but severely damaged tubules had a marked depression of the glomerular filtration rate. Subsequently Schainuck *et al* (1970) also demonstrated that impairment of renal function was most closely related to renal interstitial changes regardless of the nature of the basic kidney disease, and showed a clear inverse relationship between glomerular filtration rate and the total score for tubulo-interstitial changes. Such an inverse relationship was not demonstrated by glomerular filtration rate for any other functional index and histological glomerular injury.

This striking relationship between renal function and tubulo-interstitial disease rather than glomerular disease has now been substantiated by several authors (Bohle *et al* 1979, 1981, 1991, Park *et al* 1986, Hooke *et al* 1987, Cameron *et al* 1989, Alexopoulos *et al* 1990). It appears to be hold true for multiple renal diseases including focal

sclerosing glomerulonephritis, diabetic nephropathy, membranoproliferative glomerulonephritis, IgA nephropathy, membranous glomerulonephritis, renal amyloidosis, and chronic interstitial nephritis.

1.3 The Role of Proteinuria in the Development of Renal Scarring

1.3.1 *The relationship between proteinuria and progression of renal failure*

The early stages of the pathological process of renal scarring are detected clinically by the onset of proteinuria and sometimes haematuria. There is often a concomitant reduction in renal blood flow and glomerular filtration rate, and tubular atrophy is manifest as a failure to concentrate and acidify the urine. However of all these abnormalities the development of proteinuria is the most ominous with regard to survival of renal function.

A consistent finding in both experimental renal diseases in animals and human renal diseases is the presence of abnormal urine constituents. The most notable of these abnormal constituents is protein, and of this protein the most abundant molecule is albumin. It has been suggested that the presence of abnormal quantities of protein in the nephron may contribute to the development of renal interstitial scarring. The evidence for this assertion is based both on studies in animals and observations in human patients.

1.3.2 *Evidence from animal studies*

The histological process of renal interstitial fibrosis has been described in detail by Kuncio et al (1991). These authors described three key phases in the development of fibrogenesis in the renal interstitium: induction, matrix deposition and resolution. The phase of induction involves the recruitment into the interstitium of immune cells, activation of macrophages, stimulation of fibroblast proliferation, and dissolution of matrix. There is now good evidence that these induction events may be provoked by excess proteinuria.

A single intravenous injection of adriamycin into rats induces glomerular changes similar to the disease of focal segmental glomerulosclerosis seen in humans, and is associated with heavy proteinuria analogous to the nephrotic syndrome (Grond et al 1984). By performing serial renal biopsies on rats with adriamycin nephropathy it has been demonstrated by Bertani and his colleagues (1982, 1986) that the passage of large quantities of protein into the urinary space stimulates protein re-absorption by proximal tubular cells. Protein casts can occur in the lumen of more distal tubules, resulting in obstruction and dilatation of more proximal tubular segments. As a consequence of this, focal defects may occur in the tubular basement membrane, allowing extravasation of Tamm-Horsfall protein into the interstitium (Bertani *et al* 1986). It has been demonstrated in several studies that Tamm-Horsfall protein may interact with various immune cells *in-vitro.* Horton *et al* (1990) showed that Tamm-Horsfall protein can interact with and activate neutrophils *in vitro*, and this effect may be mediated by specific receptors for Tamm-Horsfall protein

which have been demonstrated to be present on neutrophils (Thomas *et al* 1993). In addition Tamm-Horsfall protein is able to stimulate monocytes to proliferate, and enhances the expression of several cytokines by these cells (Yu *et al* 1993). These observations therefore provide a potential link between the presence of proteinuria, the initiation of tubular epithelial damage, and the induction of an interstitial inflammatory process. Interstitial nephritis has also been described in other models of experimental proteinuria induced by a variety of means including administration of the drug puromycin aminonucleoside (Eddy and Michael 1988), 5/6 nephrectomy (Morrison 1962, Nath *et al* 1985), and immunologically mediated mesangial cell injury (Bagchus *et al* 1986).

Further powerful evidence of a link between proteinuria and interstitial inflammation is provided by studies of protein overload proteinuria (Lippman 1949, Anderson and Recant 1962, Fisher and Hellstrom 1962, Davies *et al* 1978 and 1985, Rollason and Brewer 1981 and 1984, Weening *et al* 1987, Eddy 1989). In this disease model proteinuria is induced in rats by the intraperitoneal injection of large quantities of bovine serum albumin, the proteinuria usually being evident within 24 hours of the commencement of injections. This proteinuria is composed of both homologous and heterologous albumin. Heterologous albuminuria stops within 48 hours of the last injection of bovine serum albumin, but the homologous albuminuria persists after the cessation of injections (Davies *et al* 1978).

Animals treated in this manner develop glomerular lesions manifest as degenerative epithelial cell injury with fusion and loss of foot processes, together with the development of protein droplets and cytoplasmic vacuoles (Anderson and Recant 1962, Davies *et al* 1978 and 1985, Rollason and Brewer 1981 and 1984, Weening *et al* 1987). These morphological changes in glomerular epithelial cell structure are associated with the development of large pore defects (Weening *et al* 1987) and are thought to be responsible for the development of the proteinuria. The cause of these changes however is unclear. There is no evidence of deposition of immunoglobulins or complement in the glomerulus (Davies *et al* 1978), nor is there evidence of the development of antibodies to the heterologous albumin (Muckerheide *et al* 1987, Eddy 1989).

Most studies of protein overload proteinuria have concentrated on the glomerular changes, although occasional reference has been made to tubulo-interstitial pathology (Anderson and Recant 1962, Karl *et al* 1964, Davies *et al* 1978). More recently Eddy (1989) has examined in detail events in the tubulo-interstitium of rats injected with bovine serum albumin. An acute tubulo-interstitial nephritis was described in animals treated with intra-peritoneal bovine serum albumin. The pathological lesion in the kidney interstitium was manifest initially as a macrophage infiltrate, followed by significant numbers of lymphocytes, and it's severity correlated closely with the degree of proteinuria. In addition to this cellular infiltrate in the renal interstitium, damage to proximal tubular cells was evident by their

prominent expression of vimentin intermediate filaments consistent with regeneration after injury. It was postulated that the interstitial lesion seen in this model was a result of proteinuria induced proximal tubular cell injury.

The proposition that proteinuria itself provokes renal interstitial inflammation is supported further by the observation that manoeuvres which reduce proteinuria also reduce interstitial inflammatory cell infiltration. As such, enalapril treatment of animals with puromycin aminonucleoside nephrosis ameliorates both the proteinuria and the tubulo-interstitial inflammation (Diamond and Anderson 1990) independently of any effects on the blood pressure. Administration of a low protein diet to animals with this experimental renal disease also reduces albuminuria in association with a reduction of interstitial injury (Eddy *et al* 1991). Sub-totally nephrectomised rats fed a low protein diet also demonstrate a reduction in proteinuria and interstitial infiltrate (El Nahas *et al* 1983).

1.3.3 *Evidence from human studies*

In many forms of glomerular and non-glomerular renal diseases in humans the magnitude of associated proteinuria is a strong predictor of progression. Mallick *et al* (1987) examined a cohort of patients with glomerulonephritis and showed that patents with low grade proteinuria of less than 1 g/day rarely developed progressive renal failure, but that development of the nephrotic syndrome during follow up was associated

with a relative risk of progression 129 times greater than in patients with low grade or no proteinuria.

In many of the glomerulonephritides the presence of nephrotic syndrome at the outset carries a worse prognosis in terms of renal function than does the presence of more modest levels of proteinuria, and if more than 2g of proteinuria are present beyond the first 2-3 years of follow up it acts as a reliable marker, in the majority of cases, for eventual renal failure (Cameron 1979, Cameron 1990). As such D'Amico and Vendemia (1987) in a multivariate survival analysis of patients with IgA nephropathy described the level of proteinuria as the only clinical parameter which was an independent risk factor for the development of renal failure. This finding in patients with IgA nephropathy is also supported by the work of Neelakantappa *et al* (1988).

The crucial importance of proteinuria in the development of diabetic nephropathy is well established. Incipient diabetic nephropathy is characterised by the development of microalbuminuria (Mogensen *et al* 1985-86, Mogensen 1987), and overt diabetic nephropathy by proteinuria of more than 500 mg/day. Therefore diabetics without microalbuminuria progressing to proteinuria do not develop nephropathy. Conversely when diabetic patients develop proteinuria in the nephrotic range renal function usually declines rapidly to end stage (Kussman *et al* 1976, Mogensen and Christiansen 1984).

Proteinuria is also well established as an important prognostic sign in structural renal abnormalities such as reflux nephropathy. In a study of

31 patients by Kincaid-Smith and Becker (1978) the renal function of 11 individuals deteriorated and all but one of these had proteinuria averaging 2.4 g/day. All the patients who failed to progress had no proteinuria or less than 1 g/day. In other studies of both adult and younger patients a positive correlation has been found between serum creatinine, sclerosis on renal biopsy and the level of proteinuria. Patients with reflux nephropathy who reached end-stage without exception had proteinuria at presentation (Torres *et al* 1980, El-Khatib *et al* 1987, Morita *et al* 1990).

Hypertensive nephrosclerosis is sometimes associated with heavy proteinuria. According to a number of reports the presence of heavy proteinuria in this disease is associated with a poor prognosis in terms of renal function despite good blood pressure control (Marjias *et al* 1985, Montoliu *et al* 1987).

Therefore considerable evidence exists linking the level of proteinuria with the severity of many varied renal diseases, and the likelihood of progression of these diseases to end-stage renal failure. The data discussed so far do not in themselves substantiate a causal relationship between proteinuria and progression of renal failure. However the possibility of such a relationship is strengthened by data which demonstrate the slowing or halting of such progression by manoeuvres which reduce proteinuria.

In recent years particular attention has been paid to manoeuvres which may prevent the progression of diabetic nephropathy. Much interest has been shown in the use of angiotensin converting enzyme inhibitors

(ACE I) to slow the progression of diabetic renal disease. These drugs were initially employed as anti-hypertensive agents, but the ACE I seem to confer benefits in terms of preservation of renal function over and above those provided by other anti-hypertensive agents. This effect may be due in part to the unique ability of ACE I to reverse angiotensin II mediated constriction of the efferent glomerular arteriole (Anderson *et al* 1993). That the effects of ACE I are independent of their systemic antihypertensive actions is demonstrated by their equal efficacy in non-hypertensive subjects. A number of other potential mechanisms of these ACE I effects have been proposed, including modulation of the growth factor like influences of angiotensin II on glomerular mesangial cells (Wolf and Neilson 1993), inhibition of collagen synthesis (Kagami *et al* 1994), and a reduction of interstitial extracellular matrix deposition as demonstrated by Kaneto *et al* (1994) in obstructive nephropathy and Diamond and Anderson (1990) in the puromycin aminonucleoside nephrosis model.

One common consequence of ACE I, observed both in diabetic patients and in the other experimental renal diseases mentioned above, is the ability to reduce the urinary albumin excretion. This effect is observed in both hypertensive and non-hypertensives, and raises the possibility that the beneficial effects of these agents on preservation of renal function may be related to their anti-albuminuric actions.

Many studies now support the early application of ACE I in diabetics with incipient nephropathy. Mathiesen *et al* (1991) demonstrated that captopril treated patients with type I diabetes and microalbuminuria had a

33% reduction in albuminuria over four years, whereas placebo treated individuals suffered a 60% increase in urinary albumin excretion. Similar results have been published by other authors in both insulin dependent and non-insulin-dependent diabetics (Ravid *et al* 1993, Lebovitz *et al* 1994, Viberti *et al* 1994). However the most influential study was provided by Lewis *et al* (1993). These workers randomised 409 diabetic patients with overt nephropathy and a plasma creatinine below 2.5 mg/dl, to therapy with either captopril or placebo. After four years the captopril treated patients, with an initial plasma creatinine of at least 1.5 mg/dl, had a greater than 50% reduction in their rate of creatinine increase together with a decrease in proteinuria. It was concluded that captopril protected against deterioration of renal function, and was significantly more effective than blood pressure control alone. The utility of ACE I in non-diabetic renal diseases is less clear because good studies are lacking. Nevertheless several small, short term studies suggest that in non-diabetic renal disease ACE I can reduce proteinuria whereas other antihypertensives are ineffective (Apperloo *et al.* 1991, Rosenberg and Hostetter 1991, Hannedouche *et al* 1994)

Based on these observations a number of management recommendations for the prevention of progression of renal disease have been made by two panels of experts meeting in the US (Anonymous 1994b, Striker 1995). These panels suggested that ACE I should be initiated in patients with IDDM who have persistent microalbuminuria or overt albuminuria, and that ACE inhibition may be the best chance of

slowing progression of renal failure in patients with chronic renal failure and proteinuria of greater than 3 g/24 hours.

1.3.4 *Adverse effects of proteinuria on proximal tubular cell functions*

The close correlation between proteinuria and progressive renal disease is therefore well established, and there is much evidence to support a causal relationship between proteinuria and progressive renal failure. These observations have led to the development of an hypothesis which states that the filtration of proteins, most notably albumin, together with carried molecules such as fatty acids, has a toxic effect on the proximal tubule cells. This toxicity damages the proximal tubular epithelial cells and initiates the process of interstitial inflammation and scarring (Fig. 1.1).

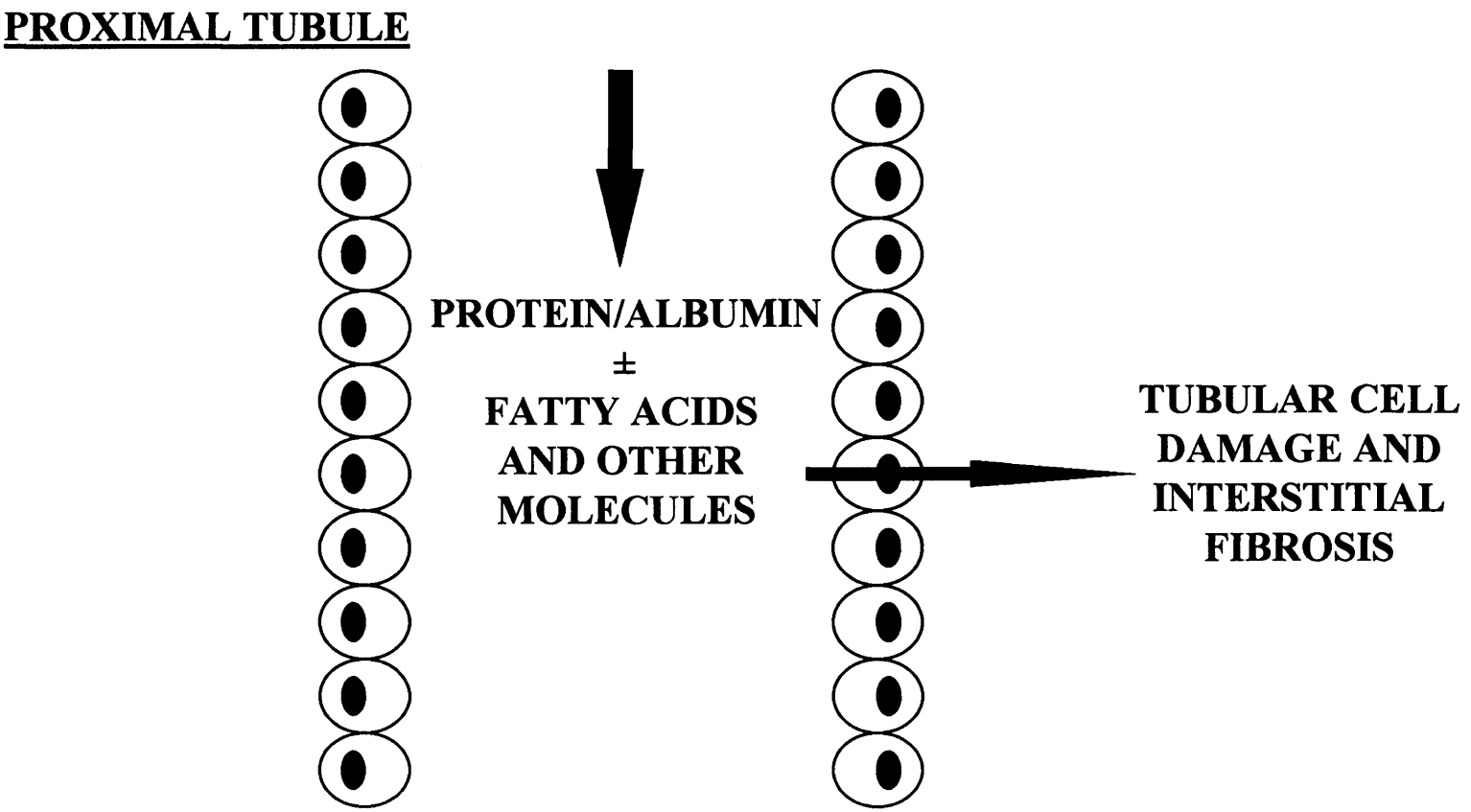
This proposal has been controversial, the conventional view being that the level of proteinuria is simply a marker of the severity of the renal lesion, such that those individuals with a more severe renal injury are more likely to exhibit progression, and that an increased level of proteinuria simply reflects the severity of the underlying injury.

However the development of interstitial inflammation as described above implies some injury to an element of the tubulo-interstitium. The predominant cell type in this area is the proximal tubular cell, and therefore it is logical to suggest that injury to these cells may initiate this inflammatory response. There is emerging evidence to suggest that proximal tubular epithelial cells are indeed capable of participating in an

Figure 1.1
Potential Mechanism of Initiation of Interstitial Inflammation

The hypothesis suggests that proteins, most notably albumin, are filtered through damaged glomeruli carrying with them other molecules such as fatty acids. These substances then exert a toxic effect on the proximal tubular epithelium thus initiating the process of interstitial inflammation and scarring.

HYPOTHESIS


PROXIMAL TUBULE
PROTEIN/ALBUMIN
±
FATTY ACIDS
AND OTHER
MOLECULES
TUBULAR CELL
DAMAGE AND
INTERSTITIAL
FIBROSIS

inflammatory process, and that such a process may be initiated by exposure of the cells to protein.

There is good evidence that proteinuria results in tubular injury. Re-absorption of lysozyme is an efficient, high capacity process which occurs in the proximal tubule after the protein is filtered trough the glomerulus (Sumpio and Maack 1982). The presence of lysozyme in the urine is a good marker of proximal tubular injury. As such in passive Heymann nephritis not only does the urinary lysozyme excretion correlate closely with urinary albumin excretion, but treatments which reduce proteinuria also reduce lysozymuria (Agarwal and Nath 1993).

The use of lysozyme as a marker of proximal tubular injury has been criticised on the basis that other proteins may compete for its re-absorption in nephrosis, and that it's appearance in the urine may simply reflect this competition for re-absorption rather than tubular cell injury. An alternative marker is the enzyme N-acetyl-β-glucosaminidase (NAG) which is present in the serum in insignificant concentrations. This protein is released into the tubular fluid by damaged proximal tubular cells and is widely used as a marker of proximal tubular injury (Guder and Hofmann 1992) since it's presence in the urine cannot be accounted for on the basis of glomerular filtration. The presence of NAG in the urine correlates well with total protein excretion in glomerular diseases (Kunin *et al* 1978, Kind 1982) and is even seen in minimal change nephropathy where during relapse of nephrosis levels of urinary NAG are high, but subsequently return to normal during remission (Kunin *et al* 1978).

Other investigators have proposed that proteinuria may exacerbate ischaemic tubular damage. For instance, in ischaemic renal injury in rats, acute tubular necrosis is made worse by concurrent administration of proteins such as myoglobin, lysozyme and ribonuclease (Zager *et al* 1987). The explanation for this phenomenon may be the further depletion of already depressed cellular energy stores, by the process of obligate tubular re-absorption of these excess low molecular weight proteins. As such, myoglobin has been shown to deplete cellular levels of adenine nucleotides in the kidney tubule (Zager 1991). There is every reason to suppose that this situation is equally applicable to the re-absorption of higher molecular weight proteins by tubular cells in renal diseases where ischaemia may not be the primary lesion.

Proximal tubular cells re-absorbing excess quantities of protein demonstrate increased lysosomal enzyme activity (Olbright *et al* 1986). It has been proposed that increased trafficking of re-absorbed proteins into lysosomes as a result of proteinuria, may lead to leakage of lysosomal enzymes into the cell cytoplasm (Maack *et al* 1971, Park and Maack 1984). The consequence of this would of course be injury to the cell and the possible initiation of interstitial inflammation.

The level of proteinuria in patients with glomerulonephritis also correlates with the level urinary ammonia excretion, and the increased ammonia generation in the kidneys of patients with renal disease has been postulated to play a role in the development of progressive interstitial

disease. This increased production of ammonia could be a result of increased catabolism of re-absorbed proteins (Nath *et al* 1985, Clark *et al* 1991, Rustom *et al* 1992). The potential importance of ammonia lies with it's ability to activate the third component of complement (Nath *et al* 1985). This phenomenon may explain the widespread peritubular and interstitial deposition of C3 and C5b→9 in proteinuric animals with elevated urinary ammonia excretion (Falk *et al* 1983, Nath *et al* 1985). These deposited complement components could serve to stimulate the release of reactive oxygen species from leukocytes, as well as acting as chemoattractants and cell activators.

1.3.5 *Pathophysiological consequences of proximal tubular cell injury by protein*

Having established the potential for tubular cell damage by proteinuria it is necessary to consider how this injury could culminate in interstitial inflammation and scarring. Tubular cells, if stimulated in an appropriate manner, are able to produce a number of pro-inflammatory cytokines. For example human tubular cells grown from renal biopsy specimens express mRNA for granulocyte-macrophage colony stimulating factor (GM-CSF), platelet derived growth factor-B (PDGF-B) and interleukin-6. The levels of Gm-CSF and PDGF-B mRNA expression were higher in those cells isolated from diseased kidneys (Frank *et al* 1993). In addition, proximal tubular cells from rodents and humans are able to produce both tumour necrosis factor-α and complement components under appropriate conditions of stimulation (Brooimans *et al* 1991, Jenvikar *et al*

1991, Yard *et al* 1992). Secreted factors such as these may enable the stimulated proximal tubular cells to attract other cell types, most notably immune cells, into the renal tubulo-interstitium and hence perpetuate the inflammatory process.

In some disease situations tubular epithelial cells also express on their surface several crucial proteins which enable them to interact with the cells of immune origin which migrate into the tubulo-interstitium. For instance, both intercellular adhesion molecule-1 and MHC class II expression has been demonstrated by proximal tubular cells in murine lupus (Wuthrich *et al* 1989 and 1990). These cells with upregulated expression of MHC class II are able to present antigen and activate T cell hybridomas (Hagerty and Allen 1992).

The above evidence indicates that proximal tubular cells, in some circumstances, may be damaged and activated in disease states such that they are able to initiate and maintain an inflammatory response. The data discussed above indicates that urinary proteins may be able to damage proximal tubular cells and thereby potentially produce a pathophysiological situation conducive to the induction of interstitial inflammation.

As well as having the capacity to induce an interstitial inflammatory response, proximal tubular cells are also able to participate in the production of fibrous scarring. It is known that proximal tubular cells may be induced to secrete matrix proteins themselves and thus promote interstitial scarring. As such Ziyadeh *et al* (1990) described increased collagen gene transcription by mouse proximal tubular cells on exposure to

high glucose concentrations. Furthermore the supernatant derived from proximal tubular cells in culture, especially those cells derived from diseased kidneys, is able to stimulate fibroblasts to produce fibronectin, a component of extracellular matrix (Frank *et al* 1992). More recent work by Burton *et al* (1994) has shown that human proximal tubular epithelial cells in culture can secrete fibronectin from their basal surface when exposed to serum at their apical membrane.

Studies by Peruzzi *et al* (1996) confirm that proximal tubular cell function may be modulated by albumin. These authors studied human proximal tubule cells in culture, and observed that the addition of albumin to the culture medium resulted in changes in the expression of adhesion molecules by the cells. Specifically expression of the $\alpha_v\beta_5$ integrin could be modulated by albumin. Expression of this integrin was also observed in the renal biopsies of proteinuric but not non-proteinuric patients.

The exposure of proximal tubular cells in culture to albumin can in some circumstances lead to enhanced cell growth. When opossum kidney cells in culture are exposed to albumin the presence of certain carried fatty acids can have diverse effects on cell growth. For instance when palmitate is complexed with albumin inhibition of growth is observed. However when albumin is complexed with oleate an opposite effect is observed, in that cell proliferation is potentiated compared to cells incubated with fatty acid free albumin (Thomas and Schreiner 1993). A similar effect was observed by Burton *et al* (1994) when opossum kidney cells were incubated with urine from nephrotic rats. Albumin was seen to enhance the proliferation of

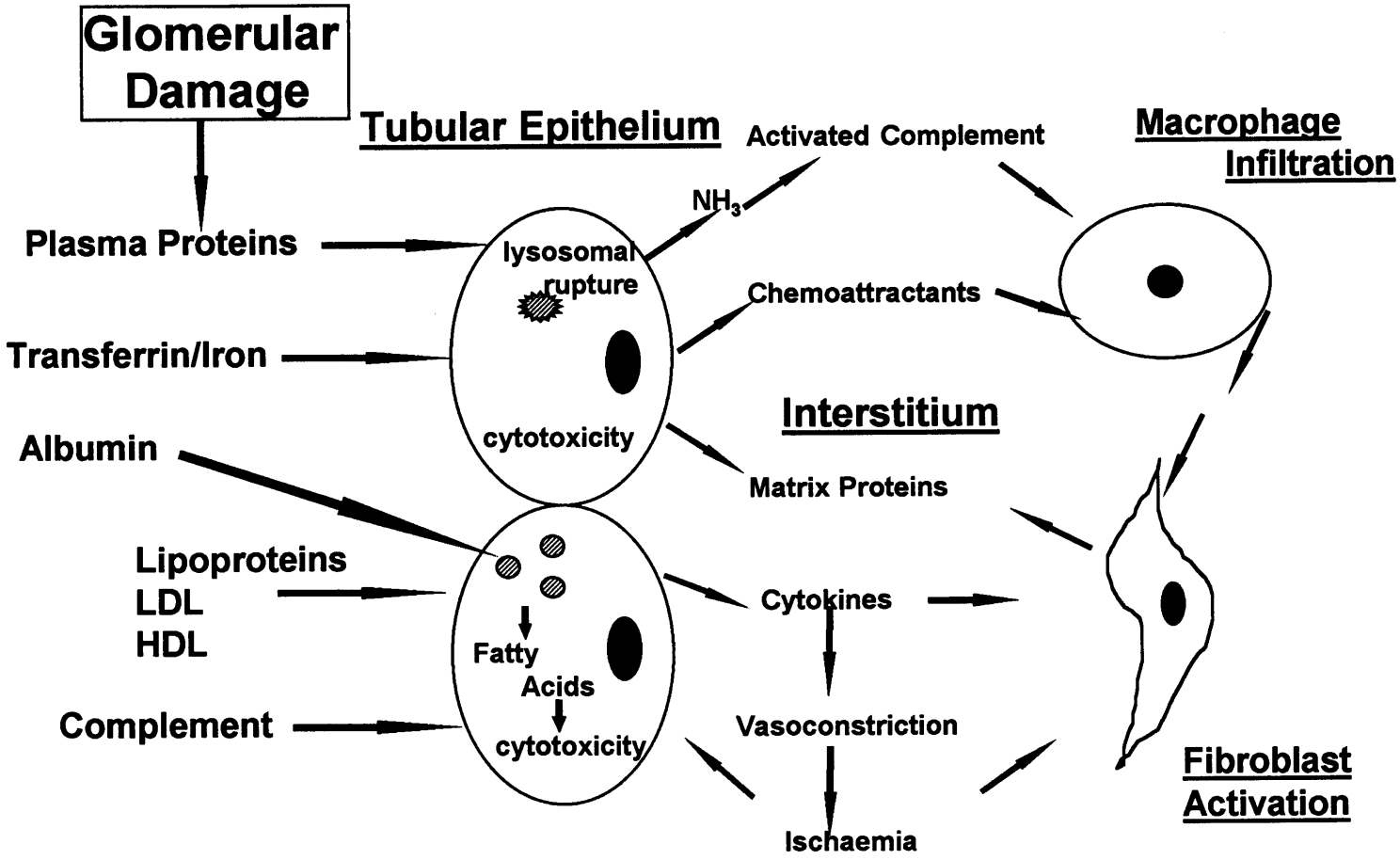
these cells, although the effect of the complete nephrotic urine was even greater.

Much recent evidence suggests that filtered lipids, and in particular those lipids bound to albumin, may play a crucial role in the pathogenesis of interstitial inflammation. The uptake of lipids by proximal tubular cells is characteristic of the nephrotic syndrome (Quinn and Zimmerman 1954, Schumann and Weiss 1981, Zimmer et al 1961, Schreiner 1971), and the uptake of fatty acids by cultured proximal tubular cells, in quantities reminiscent of nephrosis, results in major perturbations of the resting cellular lipid pool (Thomas *et al* 1995). It is probably relevant therefore that when exposed to fatty acid bearing albumin, but not fatty acid free albumin, proximal tubular segments produce a lipid chemoattractant which has been postulated to have an important role in the production of tubulo-interstitial inflammation. This lipid chemoattractant is also found in the urine of rats with protein overload proteinuria in association with the inflammatory interstitial infiltrate as already discussed (Kees-Folts *et al* 1994).

Clearly therefore a number of potentially important pathophysiological mechanisms exist whereby tubulo-interstitial inflammation may be provoked by abnormal constituents in glomerular filtrate and are summarised in Figure 1.2. Abnormal quantities of filtered proteins are likely to have a central role in this process. These observations therefore raise the possibility that by modulating the interaction of filtered protein, particularly albumin, with proximal tubule cells the detrimental effects of albuminuria may be attenuated.

Figure 1.2
Induction of Interstitial Inflammation and Scarring

This figure summarises the potential mechanisms whereby abnormal glomerular filtrate may impact on proximal tubular cell biology to initiate and perpetuate an interstitial inflammatory cell infiltrate, with activation of fibroblasts and the development of renal tubulo-interstitial scarring.


Glomerular Damage
Tubular Epithelium
Activated Complement
Macrophage Infiltration
NH_3
Plasma Proteins
lysosomal rupture
Chemoattractants
Transferrin/Iron
cytotoxicity
Interstitium
Albumin
Matrix Proteins
Lipoproteins
LDL
HDL
Cytokines
Fatty Acids
Complement
Vasoconstriction
cytotoxicity
Fibroblast Activation
Ischaemia

1.4 The Renal Handling of Proteins

1.4.1 *Filtration*

A renal glomerulus is composed of a tuft of specialised capillaries supplied by an afferent arteriole, drained by an efferent arteriole and covered by the Bowman's capsule. The vessels are formed by a fenestrated endothelium. The fenestrated regions of the endothelium are extremely attenuated and characterised by round to oval pores up to 100nm in diameter (Larsson and Maunsbach 1980). Compared to fenestrated endothelia in other sites these pores are mostly wide open and lack a diaphragm (Jorgensen 1966).

The entire tuft of capillaries is covered by epithelial cells, or podocytes. These cells represent the visceral layer of Bowman's capsule. These highly differentiated cells have a voluminous cell body which bulges into the urinary space. Long primary processes emerge from the cell body and extend towards the capillary to which they are attached by numerous secondary foot processes. The tips of the foot processes are buried within the glomerular basement membrane to a depth of about 60 nm The foot processes of individual cells interdigitate with those of adjacent cells leaving between them an intricate network of slits, known as filtration slits. The slits are narrowest at their base where they have a diameter of 25-65 nm (Bulger and Hebert 1988, Tisher and Brenner 1989). The filtration slits are bridged by an extracellular slit diaphragm with a regular subunit structure. The spaces between the subunits have an area of around 14

nm^2, approximately the size of an albumin molecule (Rodewald and Karnovsky 1974).

At the vascular pole the visceral layer of cells becomes the parietal layer which is a simple squamous epithelium. At the urinary pole the parietal epithelium of Bowman's capsule abruptly transforms into the high epithelium of the proximal tubule. The space between the two layers of the Bowman's capsule forms the urinary space, and at the urinary pole this passes into the tubule lumen.

The glomerular basement membrane is found at the interface between the glomerular capillary endothelium and the visceral epithelial podocytic layer of Bowman's capsule. This membrane develops from both the epithelial podocytes and the capillary endothelium (Abrahamson 1987). It is composed of three layers of differing electron density, a middle electron dense layer called the lamina densa bounded by two electron lucent layers, the lamina rara interna and lamina rara externa. The composition of the glomerular basement membrane is similar to that of basement membranes at other sites (Mohan and Spiro 1986, Timpl and Dziadek 1986). The major components include type IV collagens, heparan sulphate proteoglycans, and laminin (Abrahamson 1987).

The filtration barrier in the glomerulus is composed of the endothelium with it's wide open pores, the peri-capillary portion of the of the glomerular basement membrane, and the slits between the podocyte foot processes covered by the slit membrane. Therefore filtration occurs entirely via the extracellular route. The permeability characteristics of the

glomerulus are notable in that the permeability for ions and small solutes is extremely high, whereas the permeability for macromolecules, including plasma proteins, is very low.

All components of the filtration barrier can be expected to be highly permeable for water, small solutes and ions. The barrier function for macromolecules is selective for both size and charge. The entire depth of the filtration barrier represents an electronegative shield which acts to repel polyanionic substances such as plasma proteins. This electronegativity is provided by charged molecules in the surface membranes of endothelial and epithelial cells, and molecules, especially heparan sulphate proteoglycans, within the layers of the basement membrane itself (Caulfield and Farquar 1976, Kanwar and Farquar 1979). The size selectivity of the filtration barrier is provided by the dense network of the glomerular basement membrane. Uncharged molecules with a radius up to 2.0 nm are able to pass freely through the membrane. The passage of larger substances is progressively constrained until they are totally restricted at an effective radius of 4.0 nm or more. Bearing this in mind it is of interest to note that plasma albumin has a radius of 3.6 nm, and would pass through the size selective barrier in considerable amounts were it not for the additional charge barrier (Cotran and Rennke 1983, Deen *et al* 1979, Elger and Kriz 1992, Kanwar 1984, Robertson 1980).

Therefore under normal conditions in health the glomerular basement membrane provides an effective barrier to the filtration of large macromolecules into the proximal tubular fluid. However in numerous

pathological conditions the integrity of the filtration barrier is disrupted. The mechanisms of this breakdown of glomerular permselectivity have been reviewed (Savin 1993) and include toxic and immunological injury to the cells in the barrier, and neutralisation of the charge barrier. Whatever the cause, the result is the passage of much larger quantities of macromolecules, in particular proteins, into the proximal tubular fluid.

1.4.2 The fate of filtered protein

1.4.2.1 Evidence for protein re-absorption in the proximal tubule

It has been recognised for many years that proteins entering the proximal tubular fluid may be re-absorbed by the proximal tubular epithelium (Lambert 1932). Subsequently Oliver *et al* (1954) demonstrated that the hyaline droplets commonly observed in proximal tubular cells represented protein reabsorbed from the tubular lumen.

Endocytic uptake has been studied at the electron microscope level for a number of proteins including albumin (Maunsbach 1966 and 1970, Bourdeau *et al* 1972, Park and Maack 1984, Schwegler *et al* 1991), lysozyme (Christensen and Maunsbach 1976), haemoglobin (Miller 1960, Ericsson 1965), horseradish peroxidase (Graham and Karnovsky 1966, Larsson and Maunsbach 1975) and β_2 microglobulin (Maunsbach and Christensen 1976, Sundin *et al* 1994). As such considerable information is available regarding the morphological aspects of protein reabsorption in the renal tubule.

In the renal proximal tubule the endocytic pathway from the apical membrane is constituted by a number of cellular organelles: apical

endocytic invaginations, endocytic vesicles and vacuoles, dense apical tubules, and lysosomes (Christensen and Nielsen 1991). The components of this endocytic apparatus are not uniformly distributed throughout the proximal tubule. For example the lysosomes are smaller and the entire vacuolar apparatus is less well developed in the S3 segment than the S1 and S2 segments of the proximal tubule in both the rat (Christensen and Nielsen 1991) and the rabbit (Clapp *et al* 1988). Despite their morphological differences in ultrastructure, in the rabbit at least, the S1, S2 and, S3 segments of the proximal tubule each has the capacity to reabsorb albumin to the same degree. More distal segments of the nephron have an insignificant protein reabsorptive capacity (Carone *et al* 1979).

1.4.2.2 The components of the endocytic pathway

Endocytic processes can be broadly divided into fluid phase endocytosis and absorptive endocytosis. The component of protein endocytosis contributed by the fluid phase is insignificant, and probably removes less than 2% of filtered proteins (Christensen and Maunsbach 1979, Park and Maack 1984, Schiller and Taugner 1980). Therefore in the proximal tubule protein reabsorption occurs mainly by absorptive endocytosis.

The first event in absorptive endocytosis involves binding of the protein to the apical membrane of the cell. The nature of the binding sites is controversial. The electrical charge of the protein is probably of some importance in it's initial binding. It is well known that vertebrate cell membranes carry a net negative charge (Weiss 1969) and unsurprisingly

cationic proteins appear to bind preferentially to the proximal tubular cells compared to anionic forms of the same molecule (Baumann *et* al 1983, Christensen *et al* 1983, Park and Maack 1984). However anionic proteins, such as insulin and β_2-microglobulin, are also generally reabsorbed very efficiently in the proximal tubule (Chamberlain and Stimmler 1967, Petersen *et* al 1969, Nielsen *et al* 1987, Nielsen and Christensen 1989). Furthermore proteins with similar molecular weight and isoelectric point are not necessarily reabsorbed to the same degree (Christensen *et al* 1983, Nielsen *et al* 1989).

These latter observations suggest that additional cell surface factors, such as receptors, may have an important influence on protein re-absorption in the proximal tubule. Support for this hypothesis is provided by the findings of Bernard *et al* (1988). These workers challenged the concept of charge selective reabsorption of proteins in the proximal tubule, and suggested that proteins filtered through the glomeruli are reabsorbed by common tubular endocytic sites, irrespective of their physico-chemical features.

Therefore the initial event in the endocytosis of protein in the proximal tubule is binding to sites either directly in the apical invaginations, or in areas of the membrane distant from the invaginations. From here ligand bound receptors may migrate in the plane of the membrane to the apical invaginations prior to the initiation of endocytosis (Grinnell *et al* 1975, Christensen and Nielsen 1991)

Morphological studies demonstrate that the apical membrane of the proximal tubular epithelium is well equipped for endocytosis. The endocytic invaginations of the apical membrane are coated with a highly developed and elaborate clathrin lattice (Lin *et al* 1982, Rodman *et al* 1984). This lattice is more highly developed than in most cell types. The other cell types with such a highly developed clathrin coat, eg oocytes (Perry *et al* 1978) and macrophages (Aggeler and Werb 1982), also have very prominent endocytic properties. The implication of these findings is that proteins may bind to receptors which subsequently become concentrated in these clathrin coated apical invaginations prior to their internalisation by endocytosis. This has been demonstrated to be the mechanism underlying agonist mediated internalisation of muscarinic cholinergic receptors (Tolbert and Lameh 1996) and β_2-adrenergic receptors (Goodman *et al* 1996).

There is as yet no direct evidence to support the existence of clathrin coated pit mediated endocytosis of albumin bound to receptor(s) in the apical membrane of the proximal tubule. However the finding of gold labelled albumin bound predominantly in clathrin coated invaginations of proximal tubule cells prior to internalisation strongly suggests that this is indeed the mechanism responsible (Christensen and Nielsen 1991). Furthermore fluorescent dextran in glomerular filtrate is endocytosed by the proximal tubule and can be detected in clathrin coated vesicles isolated from these cells (Hammond and Verroust 1994).

1.4.2.3 The proximal tubule albumin receptor

The precise identity of the proximal tubule albumin receptor is unknown. In fact it is not yet clear whether this binding site is specific for albumin, or whether it has a more broad specificity for the re-absorption of other proteins and macromolecules.

Several groups of workers have examined both the capacity and affinity of albumin binding sites in the kidney. Early work by Park and Maack (1984) demonstrated the presence of both high affinity low capacity, and low affinity high capacity binding sites for [^{3}H]-albumin in perfused rabbit proximal tubules. Subsequently both Schwegler *et al* (1991) and Gekle *et al* (1996) have described the presence of saturable binding sites for fluorescein isothiocyanate (FITC)-labelled albumin in proximal tubule derived opossum kidney (OK) cells. Only one high affinity binding site, characterised by half maximal binding (K_M) of 20 mg/L, could be resolved from the FITC-albumin binding data obtained in these experiments. Ranganathan and Mego (1986) have investigated the binding of modified albumins to a renal cortical membrane preparation derived predominantly from basolateral membranes. Two classes of receptor were identified for both formaldehyde treated albumin and reduced-carboxymethylated albumin. These receptors had differing affinities. The high affinity receptor having a K_D 3.2 x 10^{-6} M, and the lower affinity receptor having a K_D 4.7 x 10^{-5} M. More recently attention has been focused on the role of gp330 (megalin) in albumin re-absorption in the proximal tubule. This molecule is localised along the endocytic pathway in the proximal tubule (Christensen *et al* 1995) and appears to be

responsible, at least in part, for the re-absorption of several ligands from the proximal tubule. Molecules which are thought to bind to gp330 include plasminogen activator/plasminogen activator inhibitor type I complexes (Moestrup *et al* 1993), gentamicin and polymixin B (Moestrup *et al* 1995). Cui *et al* (1996) have recently proposed that gp330 is able to bind and re-absorb albumin in the rat proximal tubule, although whether or not this occurs within the physiological range of albumin concentration was not established.

Albumin binding properties of endothelial cells have been studied in some detail by a number of authors. Although the results differ, broadly speaking it appears that endothelial cells possess discrete binding sites or receptors for both native and chemically modified albumins. The receptor for native albumin is a glycoprotein with a molecular weight of 60 kDa and has been christened "albondin" (Schnitzer 1992, Schnitzer and Oh 1994, Schnitzer *et al* 1988, 1990, Tiruppathi *et al* 1996). Putative glycoprotein receptors for modified albumins on endothelial cells have been ascribed molecular weights ranging from 14 kDa to 32 kDa (Ghinea *et al* 1988 and 1989, Schnitzer *et al* 1992, Schnitzer and Bravo 1993). There is currently no data regarding the distribution of these potential receptors in kidney cells.

1.4.2.4 Internalisation of the receptor/ligand complex

After binding of protein to the cell surface receptors and subsequent concentration in apical endocytic invaginations, the invaginations are pinched off into the cell to form small endocytic vesicles (Christensen

1982). These vesicles subsequently coalesce to form larger endocytic vacuoles. Proteins then detach from the inner coat due to acidification of the vacuole (Christensen and Maunsbach 1980). Dense apical tubules, which may be seen connected to endocytic vacuoles (Christensen and Nielsen 1991), serve to recycle reusable components, such as receptors, to the apical membrane (Christensen 1982, Van Deurs and Christensen 1984, Hatae *et al* 1986a, Hatae *et al* 1986b).

Endocytosed protein is transferred from endocytic vacuoles to lysosomes in the proximal tubule cells (Strauss 1959, Christensen and Nielsen 1991). This transfer is rapid, and intravenously injected proteins can be found in proximal tubule lysosomes as early as one minute after injection (Davidson *et al* 1971, Christensen 1976). Here proteins are broken down to their constituent amino acids, which are subsequently re-absorbed into the circulation across the basolateral membrane of the cell (Carone *et al* 1971, Park 1988). The vectorial nature of this pathway, ie apical to basal transport of proteins and metabolites, is strictly maintained. There is no evidence to suggest that degradation products of these proteins can reappear in the urine. The question of transtubular transport of intact protein is controversial. It is most likely however that large proteins like albumin are completely degraded within the cell, and that intact protein crosses the basal membrane in quantitatively insignificant amounts (Park and Maack 1984, Park 1988, Christensen and Nielsen 1991).

1.5 The Regulation of Endocytosis

The mode of uptake of protein into proximal tubule cells by endocytosis is well established as discussed above. Following the association of a protein with it's binding site this process is composed of a number of discrete steps: the migration of the ligand/receptor complex to a clathrin coated invagination, the budding of this invagination with it's complement of ligand/receptor complexes into the cell, and the trafficking of the contents of the endosomal vesicle so formed to their ultimate target. It is unclear whether the distribution of receptors for these ligands is restricted to these pits, or rather that after ligand binding the ligand/receptor complex becomes trapped when it encounters a pre-existing pit, or indeed if the ligand occupation of a receptor allows the complex to recruit soluble clathrin coat and other cytoplasmic factors that form new pits thus mediating internalisation. Recent evidence from studies with the IgE-bound high affinity Fc receptors (FcεRI) have provided some clarification (Santini and Keen 1996). These receptors are rapidly internalised via clathrin coated pits only when cross-linked by the binding of multivalent antigens. In resting RBL-2H3 cells, a continuous rat mast cell line, the FcεRI is found distributed over the entire cell membrane. However upon antigen-induced crosslinking the receptors become localised into clathrin coated pits and are rapidly cleared from the membrane. This event is not associated with any redistribution of clathrin or it's associated adapter protein 2 (AP2). These observations indicate that endocytosis results primarily from receptors migrating to pre-existing pits (Santini and Keen 1996). Whether this mechanism is applicable to

receptor internalisation in general, and albumin receptor internalisation in particular, remains to be determined.

1.5.1 *Internalisation of ligand/receptor complexes in clathrin coated pits*

One important feature of clathrin mediated endocytosis is selectivity. Certain membrane proteins, notably receptors for extracellular ligands such as low density lipoprotein, transferrin, and epidermal growth factor, are very efficiently concentrated in clathrin coated vesicles (Robinson *et al* 1996) after ligand binding. Other membrane proteins such as the glycosylphosphatidylinositol-linked Thy-1 antigen are specifically excluded (Bretscher *et al* 1980). The crucial determinant of this selectivity appears to be the presence or absence of a tyrosine containing internalisation signal in the cytoplasmic domain of the membrane receptor protein (Trowbridge *et al* 1994). This internalisation signal appears to work by binding to the AP2 adapter complex which forms the inner most layer of the coat, and binds the clathrin to the membrane (Sorkin and Carpenter 1993, Robinson *et al* 1996).

Under resting conditions some receptors, particularly those required for internalisation of nutrient transport proteins, such as low density lipoprotein and transferrin, are constitutively internalised with or without bound ligand (Goldstein *et al* 1985). Conversely, signalling receptors such as the epidermal growth factor receptor, insulin receptor, and adrenergic receptors have very low rates of constitutive internalisation (Seaman *et al* 1996). Clearly after ligand binding some form of activation occurs which

then allows the receptor/ligand complex to associate with clathrin coated pits thus facilitating internalisation. The mechanism of this activation is controversial, but studies of the epidermal growth factor and insulin receptors suggest that tyrosine autophosphorylation subsequent to ligand binding may cause a conformational change in the receptor exposing an otherwise cryptic internalisation motif (Carpentier and McClain 1994, Gilboa *et al* 1995, Lamaze and Schmid 1995, Seaman *et al* 1996).

More recent evidence has demonstrated that phosphorylation is not the only covalent modification involved in the regulated endocytosis of plasma membrane receptors. In yeast ubiquitination of the Ste2p pheromone receptor is required before internalisation can occur (Hicke and Riezman 1996). In mammalian cells tyrosine kinase activity of the epidermal growth factor receptor and c-Kit results in ligand stimulated polyubiquitination (Galcheva-Gargova *et al* 1995, Yee *et al* 1994). Whether this modification is directly recognised by the endocytic machinery or whether it results in a conformational change that exposes a sorting signal remains to be determined.

After the sorting and localisation of appropriate ligand/receptor complexes to clathrin coated pits or invaginations, these regions are pinched off into the cell to complete the internalisation. This process is accomplished by other cytosolic protein factors which associate with coated pits in addition to clathrin and AP2. Dynamin is a peripheral guanosine triphosphatase initially distributed about the surface of a nascent clathrin coated bud. It is thought that when provided with a

suitable signal, for instance the acquisition of a full complement of receptors or other physiological message, dynamin undergoes covalent modification causing it to polymerise and constrict the neck of a coated pit, eventually causing it to pinch off the cell membrane completely and enter the cell as a vesicle (Hinshaw and Schmid 1996, Schekman and Orci 1996).

It seems reasonable to speculate therefore that an albumin receptor in the proximal tubule cell may possess a similar internalisation motif, and may possibly undergo autophosphorylation and/or ubiquitination, although this remains to be confirmed.

1.5.2 *Mechanism of intracellular vesicular trafficking*

Once an intracellular vesicle has been formed it does not travel randomly around the cell with it's cargo. Instead the process of vesicular trafficking is directly targeted, and subject to regulation, to ensure that the contents of the vesicle arrive at the correct destination(s) in the correct quantities.

It is well established that after endocytosis via clathrin coated pits, internalised ligand-receptor complexes enter the endosomal compartment, a system of interconnected tubulovesicular elements (Trowbridge *et al* 1994). From this endosomal compartment many membrane components are constitutively and rapidly recycled to the plasma membrane (Mayor *et al* 1993, Trowbridge *et al* 1994), whilst the fluid phase protein in the luminal content proceeds towards the lysosome.

One of the most distinctive vacuolar structures of the endocytic pathway is the multivesicular body. During their processing in the endosome ligand-receptor complexes accumulate in these multivesicular bodies (Hopkins *et al* 1990, Felder *et al* 1990). The transfer step between the multivesicular body and the next stage in the endocytic pathway remains to be defined and has been the subject of considerable debate. The classical model suggests that multivesicular bodies undergo a maturation process, gradually losing their endosomal characteristics and acquiring the characteristics of lysosomes, such as acid hydrolases (Murphy 1991, Van Deurs *et al* 1993). More recently an alternative model proposes that multivesicular bodies are carrier vesicles that bud from the endosome and carry a selected subset of endosomal contents to a pre-existing late endosome or pre-lysosome compartment (Gruenberg *et al* 1989, Griffiths and Gruenberg 1991). Indeed recent evidence however has convincingly demonstrated that in some circumstances at least multivesicular bodies are targeted to, and can dock and fuse with pre-existing lysosomes (Futter *et al* 1996).

The mechanisms by which this targeting of vesicular traffic maintains it's accuracy and is regulated have been the subject of intense study. Many of the core components of the vesicle fusion machinery have been identified in cell free membrane fusion experiments. The first protein identified was an N-ethylmaleimide sensitive factor (NSF), a cytosolic ATPase required for transport of components between golgi cisternae (Glick and Rothman 1987, Block *et al* 1988). Subsequently it was

demonstrated by Clary *et al* (1990) that the association of NSF with membranes requires soluble proteins known as SNAPs (soluble NSF-attachment proteins). The SNAPs in turn interact with a group of membrane proteins called SNAREs or SNAP receptors (Bennett *et al* 1992, Sollner *et al* 1993). The SNAREs are divided into two groups according to, their membrane localisation, the SNAREs on vesicles being referred to as v-SNARES and those on the target membrane as t-SNARES. This complex of proteins is thought to determine the specificity of vesicular targeting, such that each transport vesicle carries a unique address marker of one or more v-SNAREs which can only interact with the appropriate t-SNARE, thus ensuring the targeting of vesicles to the correct intracellular location. These basic principles have mostly been developed during the study of Golgi vesicular transport, but are rapidly being generalised as other transport steps are reconstituted and analysed in cell free systems (Rothman 1994).

Whilst the SNAP/SNARE system of proteins ensures targeting accuracy of transport, other regulatory proteins are involved in the promotion of pairing of v-SNARES with t-SNARES. One key group of proteins are the low molecular weight guanosine triphosphate binding proteins (low molecular weight G-proteins). The Rab family of low molecular weight G-proteins proteins is particularly crucial (Novick and Brennwald 1993, Sogaard *et al* 1994). They are thought to promote pairing of v-SNARES with t-SNARES, thereby permitting close juxtaposition of the

vesicular and target organelle membrane and catalysing the fusion reaction (Rothman 1994).

1.5.3 *Heterotrimeric G-proteins in endocytic regulation*

Despite the recent advances in understanding of the mechanism of regulation of receptor mediated endocytosis much still remains to be learned. In particular there is little data available regarding regulation of endocytosis in the kidney proximal tubule.

One family of molecules which has been implicated in the regulation of protein trafficking is the heterotrimeric guanosine triphosphate binding proteins (heterotrimeric G-proteins). The observation of the dependency of adenylate cyclase activation on two regulatory ligands, hormone and guanosine triphosphate (GTP), by Rodbell *et al* (1971) and Cassel and Selinger (1976) was followed shortly afterwards by the very important discovery of proteins, discrete from the receptor and the adenylate cyclase enzyme, which were able to bind GTP and whose presence was essential for activation of the enzyme by receptor agonist (Ross and Gilman 1977, Ross *et al* 1978, Sternweiss *et al* 1981). This discovery of G-proteins revolutionised the understanding of how messages pass from the external surface of the cell to the cytoplasmic interior, and the role of these proteins as signal transducers is now well known (Sahyoun *et al* 1981, Gilman 1989, Bourne *et al* 1990, Birnbaumer *et al* 1990). Indeed it is now understood that the principles of G-protein regulation of receptor mediated adenylate cyclase stimulation are generally applicable to the receptor mediated stimulation of a great many intracellular effectors.

The heterotrimeric G-proteins are composed of 3 polypeptide subunits, referred to as α-, β-, and γ-. The α-subunit binds and hydrolyses GTP, whereas the β- and γ-subunits exist as a dimer (βγ) that serves as a functional monomer (Neer 1994). In recent years multiple members of this family of proteins have been cloned from both vertebrates and invertebrates such that at least twenty different α-subunits, five different β-subunits and twelve different γ-subunits have been identified (Hamm and Gilchrist 1996). The Gα subunits are a family of 39-52 kDa proteins which share ~45-80% amino acid homology. They have been divided on the basis of their amino acid similarity into 4 classes: Gαs, Gαi, Gαq, and Gα12 (Birnbaumer *et al* 1990, Neer 1995). The Gβ subunits are highly conserved at the amino acid level, sharing 50-83% identity, with a molecular weight of around 36 kDa. In contrast the Gγ subunits are more divergent sharing only 27-75% homology, and are much smaller with a molecular weight range of 6-9 kDa (Neer 1995, Hamm and Gilchrist 1996).

Clearly random association of these subunits could generate hundreds of different heterotrimeric complexes. In fact there are preferred combinations of isoforms which interact to form a more limited number of distinct complexes (Pronin *et al* 1992, Ueda *et al* 1994, Ray *et al* 1995, Wilcox *et al* 1995). Many of these subunits are expressed ubiquitously, whereas others have differential spatial and/or temporal expression patterns (Brunskill *et al* 1991, Andreopoulos *et al* 1995, Ryba and Tirindelli 1995). Examination of this distribution of G-protein subunits in an organ or

cell type has been useful in attempts to ascribe particular functions to an individual G-protein or cell type (Brunskill *et al* 1994).

Heterotrimeric G-proteins act as molecular switches linking ligand activated receptors to intracellular effectors. The receptors to which heterotrimeric G-proteins link are characteristic. They are serpentine in nature with seven transmembrane spanning helices. The amino terminus and three interhelical loops are exposed to the extracellular fluid, while three more loops and the carboxy terminus of the receptor are exposed to the cytoplasm (Strader *et al* 1995). Vertebrates possess approximately 1000 discrete polypeptides belonging to this family of G-protein coupled receptors, and each responds selectively to a hormone, neurotransmitter, odorant or photon, and in turn selectively activates one or more G-protein heterotrimers (Bourne 1997).

When GDP is bound to the α-subunit it associates with the G$\beta\gamma$ dimer to form an inactive heterotrimer. Following ligand stimulation of a member of the heterotrimeric G-protein linked receptor family, the receptor becomes activated resulting in a conformational change. This receptor activation causes a reduction in the affinity of Gα for GDP which then dissociates to be replaced by GTP. After GTP binding the α-subunit assumes it's activated conformation and dissociates from both the receptor and the $\beta\gamma$ dimer. The activated α-subunit is then in turn able to activate the appropriate intracellular effector. The α-subunit retains it's activated conformation until GTP is hydrolysed to GDP by the intrinsic GTPase activity of the α-subunit. Following GTP hydrolysis the α- and $\beta\gamma$-subunits

Figure 1.3
The Heterotrimeric G-Protein Activation Cycle

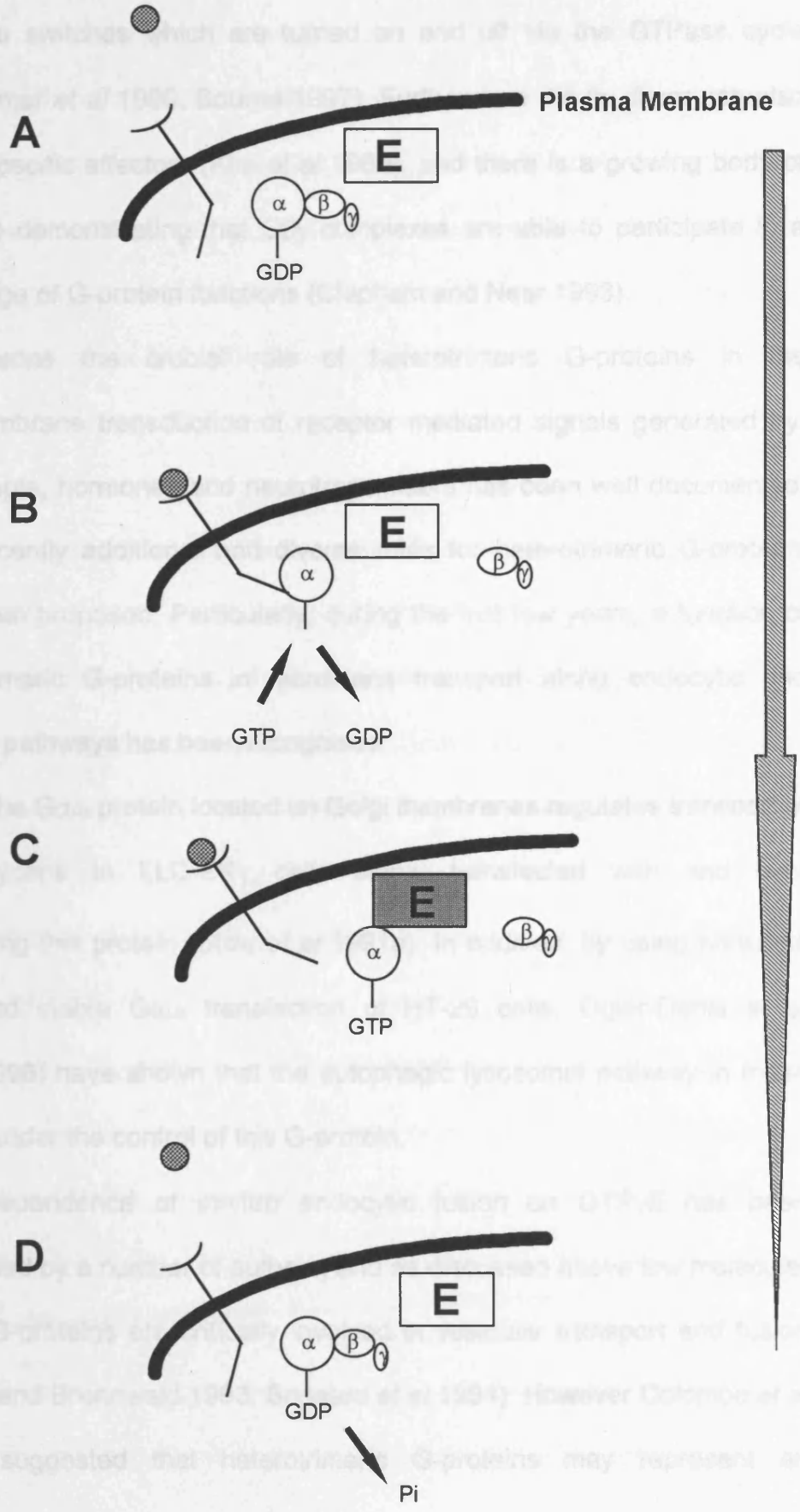
A. In the resting state the α-,β-, and γ-subunits exist as an inactive heterotrimer with GDP bound to the α-subunit. The effector (E) is also inactive.

B. When an agonist binds to the receptor in the plasma membrane a conformational change occurs in the receptor which thus interacts with Gα causing GDP to dissociate to be replaced by GTP. Gβγ also dissociate from Gα.

C. The GTP bound α-subunit is then able to activate the relevant intracellular effector (E shaded).

D. Intrinsic GTPase activity of the α-subunit hydrolyses GTP to GDP with the release of phosphate. Activation of the intracellular effector ceases, and the α-subunit reassociates with Gβγ in it's inactive form.

The Heterotrimeric G-Protein Activation Cycle



reassociate (Figure 1.3). In this way the heterotrimeric G-proteins are able to act as switches which are turned on and off via the GTPase cycle (Birnbaumer *et al* 1990, Bourne 1997). Furthermore the βγ-dimer can also act on specific effectors (Kim *et al* 1989), and there is a growing body of evidence demonstrating that Gβγ complexes are able to participate in a wide range of G-protein functions (Clapham and Neer 1993).

Hence the crucial role of heterotrimeric G-proteins in the transmembrane transduction of receptor mediated signals generated by, for example, hormones and neurotransmitters has been well documented. More recently additional and diverse roles for heterotrimeric G-proteins have been proposed. Particularly, during the last few years, a function of heterotrimeric G-proteins in membrane transport along endocytic and exocytic pathways has been recognised.

The $G\alpha_{i-3}$ protein located on Golgi membranes regulates transport of proteoglycans in LLC-PK_1 cells stably transfected with and over expressing this protein (Stow *et al* 1991a). In addition, by using pertussis toxin and stable $G\alpha_{i-3}$ transfection of HT-29 cells, Ogier-Denis *et al* (1994,1996) have shown that the autophagic lysosomal pathway in these cells is under the control of this G-protein.

Dependence of in-vitro endocytic fusion on GTPγS has been recognised by a number of authors, and as discussed above low molecular weight G-proteins are critically involved in vesicular transport and fusion (Novick and Brennwald 1993, Sogaard *et al* 1994). However Colombo *et al* (1992) suggested that heterotrimeric G-proteins may represent an

important proportion of the GTPγS sensitive factors involved in the endocytic process by demonstrating that GTPγS dependent endosome fusion could be inhibited by free G-protein βγ-subunits. Subsequently these same authors demonstrated that $G\alpha_s$ may regulate fusion among endosomes in a cell free system (Colombo *et al* 1994). Furthermore a pertussis toxin sensitive heterotrimeric G-protein regulates the association of coat components with Golgi membranes, an essential step in vesicular transport (Donaldson *et al* 1991).

In addition to regulation of endocytic processes several heterotrimeric G-proteins have been implicated in regulation of exocytosis and secretion. In polarised renal epithelial MDCK cells transport of influenza haemmagglutination protein from the *trans*-Golgi to the apical membrane is regulated by $G\alpha_s$, whereas trafficking of vesicular stomatitis virus glycoprotein to the basolateral membrane is regulated by a member of the $G\alpha_i$ family (Pimplikar and Simons 1993). Aridor *et al* (1993) described plasma membrane bound $G\alpha_{i-3}$ as an essential regulator of secretory exocytosis in mast cells. In addition, activation of a member of the $G\alpha_i$ family of G-proteins localised to insulin secretory vesicles in β-cells stimulates insulin secretion (Konrad *et al* 1995). Trafficking and delivery of the cystic fibrosis transmembrane conductance regulator from the intracellular vesicular pool to the plasma membrane in human airway epithelial cells appears to be modulated by $G\alpha_{i-2}$ (Schweibert *et al* 1995).

These observations are particularly pertinent in the context of the kidney proximal tubule since these polarised epithelial cells have large

quantities of some heterotrimeric G-proteins in their apical membranes (Brunskill *et al* 1991, Stow *et al* 1991b). This membrane domain contains in particular, large amounts of the G-protein α-subunit $G\alpha_{i\text{-}3}$, and because this apical cell domain is not thought to have a major role in hormonal signalling, the functions of such large quantities of G-proteins are not immediately apparent. It is therefore conceivable that these G-proteins may have novel functions, and one potential role may be in regulation of the endocytosis of macromolecules from proximal tubular fluid.

1.5.4 *Other enzymatic regulators of endocytosis*

Regulation of cellular function is often achieved by covalent alteration of the structure of protein molecules within the cell by the activity of enzymes. Very frequently this modification involves the addition or removal of phosphate groups from either serine/threonine or tyrosine residues, ie phosphorylation and de-phosphorylation. This process of phosphorylation and de-phosphorylation, by kinases and phosphatases respectively, provides a switching mechanism analogous to the G-protein system (Krebs and Beavo 1979).

Studies by several authors have implicated the activities of kinase and phosphatase enzymes in the regulation of both exocytosis and endocytosis. In MDCK cells polarised transport form the Golgi apparatus to the plasma membrane is inhibited by the phosphatase inhibitor okadaic acid, and is stimulated by the kinase inhibitor staurosporine (Brewer and Roth 1995). Endocytic events can also be inhibited by okadaic acid (Lucocq *et al* 1991), apparently due the ability of okadaic acid to prevent

endosomal fusion (Woodman *et al* 1992). The use of these reagents however does not allow the identification of the precise enzymes responsible for the observed effects.

Enhancement of internalisation of receptor molecules by the protein kinase C (PKC) activating phorbol esters has been demonstrated in some cells (Backer and King 1991), and indeed the transcytosis of polymeric immunoglobulin A appears to be enhanced by phorbol ester dependent phosphorylation of the cytoplasmic tail of the receptor (Cardone *et al* 1994). Furthermore a complex relationship between phorbol ester stimulation and okadaic acid inhibition of fluid phase endocytosis has been described by Sato *et al* (1996). In addition to endocytic regulation by serine/threonine phosphorylation there is also evidence that tyrosine phosphorylation may, in some circumstances, control the endocytosis of asialoglycoproteins by hepatocytes (Holen *et al* 1995a and 1995b). Clearly therefore the state of phosphorylation of some intracellular proteins appears to regulate endocytosis in many settings. The action of PKC may be important in this regard, but currently available data does not yet allow the assignment of a precise role in vesicular trafficking to this, or any other kinase.

Another enzyme whose function has been implicated in the control of vesicular trafficking is phospholipase D (PLD). This enzyme hydrolyses phospholipids into phosphatidic acid and their respective polar head group. The PLD enzyme may be stimulated by ADP-ribosylation factor, a small GTP dependent regulatory protein involved in the regulation of

various secretory pathways (Brown *et al* 1993, Cockcroft *et al* 1994), suggesting that PLD too is involved in secretory control. Several lines of evidence also suggest that phosphatidic acid, released by the action of PLD, may modulate binding of important coat proteins to the Golgi complex membranes and thus the formation of secretory vesicles (Ktistakis *et al* 1995 and 1996).

This interesting data therefore raises the question as to whether PLD may be able to regulate trafficking in the endosomal pathway in addition to the secretory pathway. It is not yet possible to answer this question, but other circumstantial evidence suggests that PLD may indeed regulate endocytosis. The potential importance of PKC in endocytic regulation was discussed above. It may be relevant in this regard therefore that PLD activity can be stimulated by PKC through it's regulatory domain rather than it's catalytic domain, ie independently of the kinase activity of the enzyme (Fabbri *et al* 1994, Simon *et al* 1996).

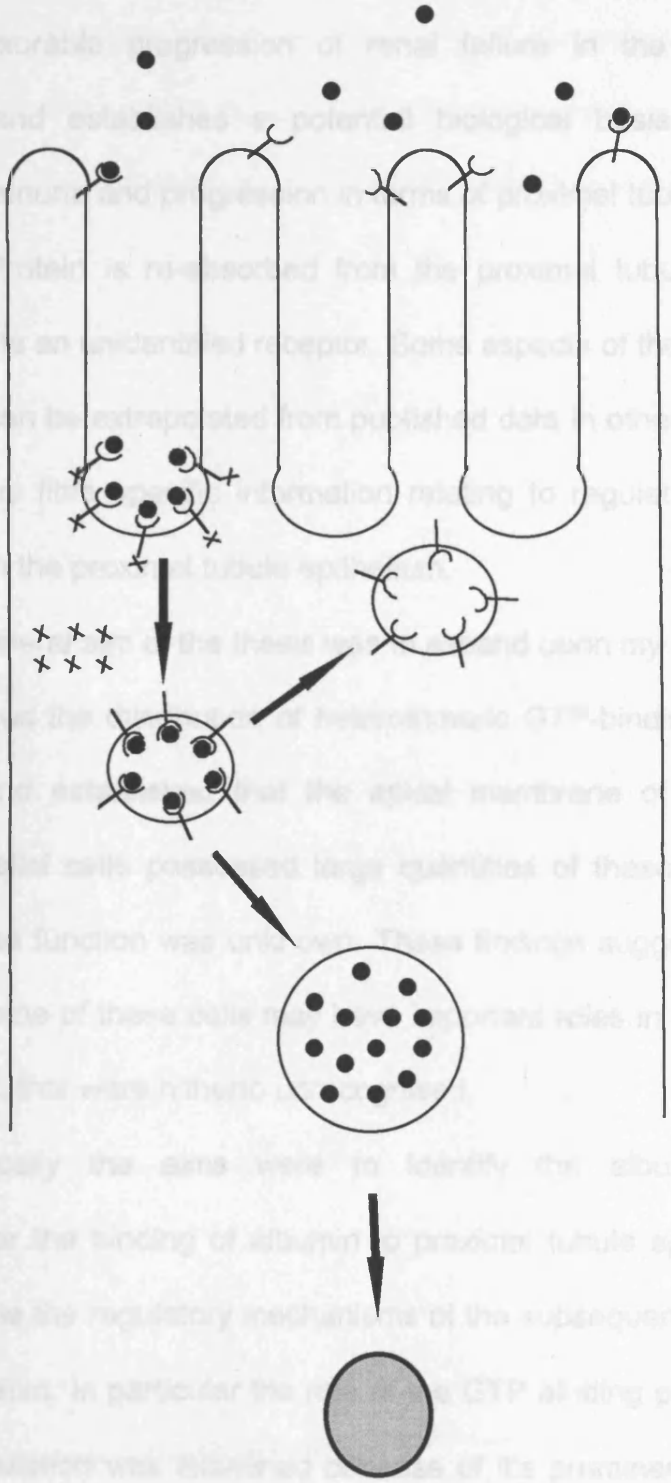
1.6 Summary

Clearly therefore the uptake of filtered protein into proximal tubular cells is a complex process. A number of discrete steps are involved which include binding ,of proteins to cell surface receptors, internalisation, vesicular trafficking and targeting, and eventual lysosomal breakdown. These events are summarised in Figure 1.4. The complexity of this process means that there are multiple points in the pathway which are potentially subject to cellular control and regulation, and which may be potentially be amenable to pharmacological modulation.

Figure 1.4
The Proposed Mechanism of Receptor Mediated Endocytosis of Macromolecules in the Proximal Tubule

Filtered protein (black dots) is filtered through a damaged glomerulus and enters the proximal tubular lumen where it binds to receptors in the apical villous membrane. These ligand bound receptors become concentrated in apical invaginations which are coated with clathrin (x). The apical invaginations are pinched off into the cell, the clathrin is released, and the receptors are recycled back to the apical membrane. The endocytosed protein enters an intracellular vesicular trafficking pathway which culminates in it's breakdown in the lysosome.

Receptor Mediated Endocytosis in the Proximal Tubular Epithelium



1.7 Aims of the thesis

The discussion above describes a problematic clinical scenario involving inexorable progression of renal failure in the presence of proteinuria, and establishes a potential biological basis for the link between proteinuria and progression in terms of proximal tubular toxicity of proteinuria. Protein is re-absorbed from the proximal tubular lumen by endocytosis via an unidentified receptor. Some aspects of the regulation of endocytosis can be extrapolated from published data in other systems, but as yet there is little specific information relating to regulation of protein endocytosis in the proximal tubule epithelium.

The general aim of the thesis was to expand upon my previous work which examined the distribution of heterotrimeric GTP-binding proteins in the kidney and established that the apical membrane of the proximal tubule epithelial cells possessed large quantities of these GTP-binding proteins whose function was unknown. These findings suggested that the apical membrane of these cells may have important roles in cell signalling and regulation that were hitherto unrecognised.

Specifically the aims were to identify the albumin receptor responsible for the binding of albumin to proximal tubule epithelial cells, and to examine the regulatory mechanisms of the subsequent endocytosis of bound albumin. In particular the role of the GTP binding protein $G\alpha_{i\text{-}3}$ in endocytic regulation was examined because of it's prominent localisation to the apical membrane domain of the proximal tubular epithelium. Experiments were also designed in order that the possible involvement of

other enzymes in endocytic regulation, particularly protein kinases and phospholipases, could be examined.

The ultimate goal of these studies is of course to gain further understanding of the pathophysiological effects of protein on the proximal tubule and to suggest therapeutic strategies for the abrogation of this tubular toxicity.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents and Antibodies

Cell culture media, additives, reagents, antibiotics and plasticware were from GIBCO Life Technologies (Paisley, UK). Nitrocellulose membranes were from BioRad (Hemel Hempstead, UK). Polyclonal rabbit anti-$G\alpha_{i-3}$ antibody EC/2 was obtained from DuPont-NEN (Stevenage, UK), goat anti-rabbit peroxidase linked second antibodies were obtained from Sigma (Poole, UK). All lectins and their cognate hapten sugars were also from Sigma (Poole, UK). Pre-stained Rainbow molecular weight standards, and an enhanced chemiluminescent (ECL) second antibody detection system were from Amersham International (Little Chalfont, UK).

The eukaryotic expression vector pcDNA3 was from Invitrogen (San Diego, CA, USA), and the rat cDNA encoding the $G\alpha_{i-3}$ subunit was kindly provided by Dr. M. Linder (Southwestern Medical Center, Dallas, TX, USA). All restriction endonuclease enzymes were from Pharmacia Biotech (St. Albans, UK). All other chemicals were from Sigma (Poole, UK) and were the highest grade available.

2.2 Radiochemicals

In order to obtain large quantities of radiolabelled albumin of consistent quality and purity [^{125}I]-human albumin was obtained from Amersham International. This preparation was supplied in sterile isotonic saline at an albumin concentration of 20 g/L, specific activity 50 μCi/ml.

[^{3}H]-inulin, specific activity 1.67 Ci/mmol, was also obtained from Amersham International.

2.3 Cell Culture

2.3.1 *Choice of cell type*

These studies necessitated the use of a kidney proximal tubular cell line and two approaches were possible. The first envisaged the use of primary cultures of human proximal tubular cells derived from nephrectomy specimens. However these primary cell cultures only retain differentiated proximal tubular cell features for a few passages, and as such it was felt that this approach would be unlikely to yield sufficient cells for the albumin uptake and binding experiments. Furthermore non-immortalised primary cell cultures are not suitable for the establishment of stable gene transfections.

The alternative approach was to use an immortalised cell line. There are three commonly used immortalised, polarised renal tubular epithelial cell lines; Madin Darby Canine Kidney (MDCK) cells, LLC-PK_1 cells derived from the pig (Hull *et al* 1976), and opossum kidney (OK) cells derived from the American opossum (Koyama *et al* 1978). MDCK cell possess characteristics reminiscent of distal tubular segments (Valentich 1981), whereas both LLC-PK_1 and OK cells have characteristics of the proximal tubular epithelium (Koyama *et al* 1978, Cantiello *et* al 1986, Malstrom *et al* 1987, Nakai *et al* 1987). However despite having many similar characteristics these two cell types are not identical (States *et al* 1991). Therefore the initial approach in this work was to use both LLC-PK_1

and OK cells in the preliminary albumin uptake experiments in order to establish which cell type was most suitable for the work (see below). On the basis of these preliminary studies it was determined that OK cells provided the most suitable model for further study.

2.3.2 *Cell culture media and incubation conditions*

All cultured cells were maintained in Dulbecco's modified Eagle's medium-Ham's F12 mix (DMEM-F12) supplemented with 10% foetal calf serum, 100 u/ml penicillin, and 0.1 mg/ml streptomycin. The cells were incubated at 37°C in an humidified atmosphere of 95% air-5% CO_2. Culture medium was changed three times a week.

2.3.3 *Passaging of cultured cells*

Cultured cells were passaged at confluence, approximately once per week, using a 0.25% trypsin, 1mM EDTA solution. Cells were then re-suspended in warmed DMEM-F12 at a dilution of 1:10. Using these conditions cells usually achieved confluence every 7-10 days. The cells in these experiments were used from passage 65 to passage 87.

2.4 Preparation of Cell Membranes

For preparation of membranes with the preservation of cell surface expressed proteins confluent OK cells growing in 80 cm^2 tissue culture flasks were harvested in a solution composed of 10 mM HEPES, 154 mM NaCl, 0.54 mM EDTA, pH7.3, and re-suspended in an ice cold homogenisation buffer composed of 50 mM Tris-HCl, 10 mM EDTA, 10 μg/ml aprotinin, 1 mM phenylmethylsulphonylfluoride, pH 7.4. The cells were homogenised with 15 strokes of a teflon pestle in a hand held glass

homogeniser. This homogenate was then spun at 20,000 rpm (30,000 g) for 20 minutes in a Sorvall RC-5C refrigerated centrifuge at 4°C. Membranes were then re-suspended in homogenisation buffer and a stored in aliquots at -80°C.

2.5 Polyacrylamide Gel Electrophoresis of Proteins

Prior to polyacrylamide gel electrophoresis (PAGE), membranes suspended in homogenisation buffer (above) were added to an equal volume of 2x gel loading buffer of (final concentrations) 50 mM Tris (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol, 5% 2-mercaptoethanol, followed by immersion in a boiling waterbath for 3 minutes. Membrane proteins in loading buffer were then applied to 12% polyacrylamide gels and electrophoresed at 200V for approximately 45 minutes according to the method of Laemmli (1970). At least one lane of each gel was reserved for the separation of pre-stained molecular weight standards in order to subsequently determine the molecular weights of proteins of interest in the OK cell membranes.

2.6 Immunoblotting of Proteins after PAGE

Separated proteins were transferred from polyacrylamide gels to nitrocellulose membranes in a semi-dry transfer cell according to the method of Towbin *et al* (1979). The polyacrylamide gel was placed on a nitrocellulose membrane and sandwiched between 3 sheets of blotting paper on each side. The whole gel sandwich was soaked in transfer buffer composed of 39 mM glycine, 48 mM Tris base, 0.037% sodium dodecyl sulphate, 20% methanol, and the transfer performed by the application of a

current of 0.65 mamps/cm^2 for 60 minutes at room temperature, with the nitrocellulose membrane orientated towards the anode, and the gel towards the cathode. Efficacy of transfer was confirmed by assessing the transfer of the pre-stained molecular weight standards from the gel to the nitrocellulose membrane.

Nitrocellulose membranes were then washed for 5 minutes in Tris buffered saline (TBS) 20mM Tris base pH 7.5, 100mM NaCl, followed by washing in TBS with 0.05% Tween-20 (TTBS) for a further 5 minutes. Membranes were blocked by immersion in 5% milk in TTBS for 1 hour at room temperature. The membranes were then incubated with anti-$G\alpha_{i-3}$ antibodies diluted 1:1000 in blocking solution for 1 hour at room temperature. Following this incubation with primary antibody the membranes were washed in TTBS 3x5 minutes, and then incubated with goat anti-rabbit peroxidase- conjugated secondary antibodies diluted 1:5000 in blocking solution for 1 hour at room temperature. Following this incubation the membranes were again washed 3x5 minutes in TTBS. After the final wash ECL reagents were applied to the nitrocellulose membrane for 60 seconds and then drained off. The membrane was then wrapped in saran wrap and exposed to photographic film for approximately 5 minutes in order to detect bound antibodies. Where appropriate bands were quantified by laser densitometry and analysed using Molecular Analyst computer software.

2.7 Aibumin Binding Studies

2.7.1 *Time course and saturation binding of [^{125}I]-albumin*

For [^{125}I]-albumin binding studies OK cells were grown to confluence in 6 well plates (Nunc, Roskilde, Denmark). Prior to the commencement of binding, the cells were quiesced in serum free DMEM F-12 for two hours and then cooled to 4°C for 30 minutes. For time course binding studies the cell monolayers were then incubated with a binding solution of 50mg/L [^{125}I]-albumin diluted in pre-cooled serum free DMEM F-12 (1ml) at 4°C for varying times. For saturation binding experiments OK cell monolayers were incubated with varying concentrations of [^{125}I]-albumin diluted in pre-cooled serum free DMEM F-12 at 4°C overnight. After the completion of binding monolayers were washed with 3x2ml ice cold phosphate buffered saline (PBS) pH 7.4 and extracted with 1 ml of 1 M NaOH. An aliquot of this cell extract was counted in an RIA Gamma Counter (Wallac, Turku, Finland), and a further aliquot taken for protein determination. Non-specific binding of [^{125}I]-albumin to cell monolayers was defined by adding of 100 g/L unlabelled human albumin to the binding solution. In preliminary experiments the adequacy of the washing protocol was checked by examining the radioactivity in an aliquot of PBS from each the washing steps to ensure that the three washes were sufficient to remove all free radioactivity from the wells.

2.7.2 *Lectin competition experiments*

For lectin competition experiments OK cell monolayers were pre-incubated with various lectins, each at a concentration of 50 μg/ml, in serum free DMEM F12 for 1 hour at 4°C prior to the initiation of [^{125}I]-albumin binding overnight at 4°C as described above. For those lectins

which demonstrated inhibition of [^{125}I]-albumin binding to OK cell monolayers in preliminary experiments, the concentration dependence of lectin inhibition of [^{125}I]-albumin binding was established by varying the concentration of lectin incubated with the OK cell monolayers from 25 μg/ml to 200 μg/ml. In other experiments lectins were pre-incubated with their appropriate hapten sugar in DMEM F12 for 60 minutes at room temperature prior to addition of the mixture to OK cell monolayers at 4°C, and the initiation of [^{125}I]-albumin binding. A range of lectins, each with a different sugar specificity, was used. The following lectins were employed in these experiments [their abbreviations and sugar specificities are shown in parentheses (together with cognate hapten sugars)]: Limulus polyphemus [LP; N-acetylneuraminic acid (N-acetylneuraminic acid)], Bandeiraea simplicifolia II [BS II; N-acetyl-D-glucosamine (N-acetyl-D-glucosamine)], Arachis hypogaea [AH; β-D-galactose-(1→3)-N-acetyl-D-galactosamine (D-galactose)], Concanavalin A [Con A; α-D-mannose > β-D-glucose (D-mannose)], Glycine max [GM; N-acetyl-α-galactosamine and N-acetyl-β-galactosamine >> α- and β-galactose (N-acetylgalactosamine)], Ulex europaeus I [UE ; α-L-fucose (L-fucose)].

2.7.3 *Other competitors of [^{125}I]-albumin binding*

In order to determine the specificity of any potential albumin receptor a number of other competitors were employed in [^{125}I]-albumin binding studies. These competitors are most conveniently divided into two classes. The first is a group of proteins which are found in normal or inflamed urine, and may therefore be in a natural position to compete for a

protein receptor in the proximal tubule. The proteins employed in these experiments were immunoglobulin, lysozyme, glycated albumin, and α_1-acid glycoprotein. The second class of competitors comprised a group of polyanionic macromolecules which have been previously documented to inhibit the binding of albumin and other ligands to scavenger receptors. These competitors included heparin, polyglutamic acid, dextran sulphate, and fucoidan. In these competition experiments the OK cell monolayers were incubated overnight at 4°C with 150 mg/L [^{125}I]-albumin in DMEM F12 in the presence of the indicated competitor at either a 10 fold or 100 fold molar excess. Cell monolayers were then washed, extracted and counted as described above.

2.7.4 *Data analysis*

Saturation binding data were analysed by non-linear curve fitting and transformed using GraphPad Prism software (San Diego, California, USA). In all binding experiments data were fitted simultaneously to both one and two site binding equations and the results compared. In all experiments the data fit best to the two site model. In some instances data were transformed and displayed as Scatchard plots for the sake of clarity, but this transformation was not employed for the analysis of data.

2.7.5 *Statistical analysis*

Where appropriate, data are represented as means ± standard error of n determinations. Where multiple comparisons were required, statistical analysis was performed by one-way analysis of variance and, for $p < 0.05$,

the significance of difference between individual group means was determined with Duncan's multiple range test at $p < 0.05$ and $p < 0.01$.

2.8 Ligand Blotting of Opossum Kidney Cell Membrane Proteins with [^{125}I]-Albumin

2.8.1 *Detection of [^{125}I]-albumin binding proteins in OK cell membranes*

For ligand blotting experiments OK cell membrane proteins were separated by PAGE and transferred to nitrocellulose membranes as described above. After transfer the nitrocellulose membranes were washed in PBS pH 7.4, at room temperature and then blocked with 3% gelatin in PBS overnight at room temperature. Following further washing with PBS 3x5 minutes the nitrocellulose membranes were incubated with 150 mg/L [^{125}I]-albumin in PBS at room temperature for 2 hours. After this the membranes were extensively washed (5x5 minutes in PBS) at room temperature and then dried and autoradiographed. The molecular weights of radiolabelled proteins were determined by comparison with co-migrating standards.

To ascertain whether the lectins would inhibit [^{125}I]-albumin binding to OK cell proteins on nitrocellulose membranes, the ligand blotting experiments detailed above were carried out with the following modifications. After the gelatin blocking step nitrocellulose membranes were incubated with a lectin at 50 μg/ml in PBS for one hour at room temperature. Following this step the nitrocellulose membranes were then incubated with [^{125}I]-albumin in PBS containing the appropriate lectin for 2

hours at room temperature. Nitrocellulose membranes were then handled exactly as described above.

2.8.2 *Concentration dependency of [^{125}I]-albumin binding in ligand blots*

The aim of these experiments was to determine whether [^{125}I]-albumin bound to the binding proteins identified by ligand blotting with affinities similar to those calculated for [^{125}I]-albumin binding to intact OK cell monolayers. Separated OK cell membrane proteins on nitrocellulose membranes were incubated with various concentrations of [^{125}I]-albumin. Identical quantities of OK cell membrane proteins were present in each lane. These nitrocellulose strips were then washed, dried and autoradiographed exactly as described above.

The intensity of labelled protein bands was measured by laser densitometry. The intensity of the labelled bands observed after incubation with the lowest concentration of [^{125}I]-albumin were arbitrarily ascribed a value of 1. The densitometry values thus determined were used to construct saturation binding curves and the data analysed using non-linear curve fitting as described above.

2.9 Lectin Blotting of Opossum Kidney Cell Membrane Proteins

In order to determine whether the lectins which inhibited [^{125}I]-albumin binding to OK cell monolayers would bind to the proteins to which [^{125}I]-albumin bound in the ligand blotting experiments, OK cell proteins were separated by polyacrylamide gel electrophoresis and transferred to

nitrocellulose membranes as described above. These nitrocellulose membranes were then incubated with the relevant peroxidase labelled lectins at a concentration of 25 μg/ml in PBS for 1 hour at room temperature. Some nitrocellulose membranes were incubated with peroxidase labelled control lectins also at a concentration of 25 μg/ml in PBS. Following the lectin incubations nitrocellulose membranes were washed extensively with PBS and bound lectin was detected using a the ECL detection system followed by autoradiography. This lower 25 μg/ml concentration of lectin was used in these experiments because higher concentrations gave unacceptable levels of background staining.

2.10 The Assay of Endocytosis

2.10.1 *Receptor mediated endocytosis of albumin*

Endocytosis of albumin was measured using [^{125}I]-albumin as a marker. On the morning of the experiment confluent OK cells growing in 6 well plates were changed to serum free DMEM F12 and quiesced in this medium for 2 hours at 37°C. The [^{125}I]-albumin uptake studies were performed in DMEM F12. [^{125}I]-albumin was added to this uptake solution at various concentrations and incubated with the OK cells at 37°C for 15 minutes. Incubations were terminated by rapid removal of the uptake solutions followed by washing with 3x2ml ice cold PBS pH 7.4. The cell monolayers were then lysed with 1 M NaOH. The amount of albumin bound to and taken up by the cells was determined by counting the cell extract in a gamma counter. An aliquot of the cell extract was saved for protein estimation.

In some experiments the time course of albumin uptake was measured by incubating OK cells with [^{125}I]-albumin for varying times prior to washing and extraction. Values for non-specific binding of albumin to OK cells were assessed by co-incubation of [^{125}I]-albumin with 100 g/L unlabelled human serum albumin.

2.10.2 *Fluid phase endocyotsis*

[^{3}H]-inulin was used as a marker for the measurement of fluid phase endocytosis in OK cells. Confluent OK cell monolayers were quiesced in serum free DMEM F12 for 2 hours at 37°C and then incubated with various concentrations of [^{3}H]-inulin in serum free DMEM F12 for 15 minutes at 37°C. Uptake was terminated by washing with ice cold PBS (3x2ml), cell monolayers were extracted with 1 M NaOH, and an aliquot of the extract neutralised and counted by liquid scintillation spectroscopy.

2.11 Visualisation of Endocytosis by Electron Microscopy

OK cells were grown to confluence on sterile glass coverslips in 24 well plates, and quiesced in serum free DMEM-F12 for 2 hours prior to the commencement of the experiments. The media was then removed and replaced with serum free DMEM-F12 containing 50 mg/L bovine serum albumin conjugated to 10 nm colloidal gold particles (gold-albumin), and allowed to incubate for 15 minutes at 37°C. As a negative control some coverslips were incubated with gold-albumin together with a 1000 fold excess of unlabelled albumin. Uptake was stopped by washing with ice cold PBS. Cell monolayers were flooded with a fresh fixative consisting of 3% gluteraldehyde in 0.1M phosphate buffer, pH 7.4 at 4°C for 3 hours.

Following fixation cells were rinsed with phosphate buffer , pH 7.4 containing 7% sucrose (3 x 30 minutes). The cells were post-fixed for 1 hour in aqueous 2% osmium tetroxide at 4°C, dehydrated in graded ethanol solutions and embedded in the epoxy resin Araldite. Upon polymerisation the glass coverslip was digested with hydrofluoric acid. Ultra-thin sections (70 - 90nm; silver to gold in interference colour) were cut using a diamond knife (Diatome) on a Reichart Jung OMU-4 ultramicrotome and contrasted with uranyl acetate and lead citrate and examined in a Jeol 100CX electron microscope using 80 KeV acceleration voltage.

2.12 Modulation of Vesicular Transport by N-Ethylmaleimide

The potent cysteine alkylating agent N-ethylmaleimide (NEM) has a number of important intracellular targets. In order to assess the effect of pre-treatment of OK cells with this agent on albumin endocytosis, the cells were pre-incubated with various concentrations of NEM in serum free DMEM F12. Pre-incubation was for 10 minutes and after this period endocytosis of [^{125}I]-albumin was allowed to proceed for 15 minutes as described above. NEM was maintained in the incubation solution throughout the period of uptake. Cell monolayers were then processed as described above.

2.13 Specific Modulation of GTP-Binding Protein Function

The functions of several heterotrimeric GTP-binding proteins were modulated by pre-treating the cells with the ADP-ribosylating bacterial toxins; cholera toxin or pertussis toxin. In the studies with bacterial toxins

OK cells were pre-treated with 500 ng/ml cholera toxin or 100 ng/ml pertussis toxin by addition to the culture medium overnight prior to the subsequent [^{125}I]-albumin uptake experiments performed as described above. Toxins were also present in the serum free DMEM F12 used to quiesce the cells on the morning of the experiments.

In order to exclude any potential interaction of the pertussis toxin with [^{125}I]-albumin binding to OK cell monolayers, control cells and cells pre-treated with pertussis toxin were incubated with [^{125}I]-albumin at 4°C to prevent internalisation. These cells were processed in exactly the same manner as those used to assess [^{125}I]-albumin uptake as described above.

2.14 GTP-Binding Protein cDNA Transfections

2.14.1 *Choice of GTP-binding protein*

The results of the experiments designed to measure albumin endocytosis after modulation of GTP-binding protein function suggested that the process of albumin endocytosis in OK cells was indeed subject to regulation by GTP-binding proteins. Therefore on the basis of previous studies, and the results of the pertussis toxin pre-incubation experiments, it was established that the most likely GTP-binding protein candidate for the regulation of albumin endocytosis was $G\alpha_{i\text{-}3}$. Therefore the cDNA encoding this protein was obtained for the purposes of establishing stable transfection in wild type OK cells.

2.14.2 *Choice of plasmid vector*

The general aim of the transfection studies was to achieve high level expression of recombinant $G\alpha_{i\text{-}3}$ protein in OK cells and to examine

the effect of this overexpression on albumin endocytosis. Therefore it was necessary to achieve stable transfection of the OK cells with $G\alpha_{i-3}$ cDNA. The plasmid vector chosen to facilitate this was pcDNA3 (Figure 2.1). This vector has promoter sequences from the immediate early gene of the human cytomegalovirus to allow high level transcription. It also has polyadenylation signal and transcription termination sequences from the bovine growth hormone gene to enhance RNA stability. Furthermore pcDNA3 has a versatile multiple cloning site with particularly convenient restriction sites for the subcloning of the $G\alpha_{i-3}$ cDNA.

2.14.3 *Subcloning of DNA*

The $G\alpha_{i-3}$ cDNA was provided by Dr M. Linder (Southwestern Medical Center, Dallas, Texas, USA) in the plasmid vector NpT7-5 as a *Hind* III/*EcoR* I insert. The plasmid/$G\alpha_{i-3}$ construct was supplied in a glycerol stock of E. coli strain BL21-DE3. The plasmid NpT7-5 is useful for protein expression and purification from bacterial cultures, but is unsuitable for transfection of genes into eukaryotic cells. Furthermore the multiple cloning site of NpT7-5 does not contain restriction sites which would allow direct excision of the $G\alpha_{i-3}$ cDNA insert followed by immediate subcloning into the chosen plasmid vector pcDNA3. Therefore the subcloning strategy was to excise the $G\alpha_{i-3}$ cDNA from BL21-DE3 using a *Hind* III/*EcoR* I digest, followed by ligation into the *Hind* III/*EcoR* I site of pBSII SK (Figure 2.2). Then the $G\alpha_{i-3}$ cDNA was excised from pBSII SK using an *EcoR* I/*Xho* I digest. This cDNA fragment could then be subcloned into the *EcoR* I/*Xho* I site of pcDNA3.

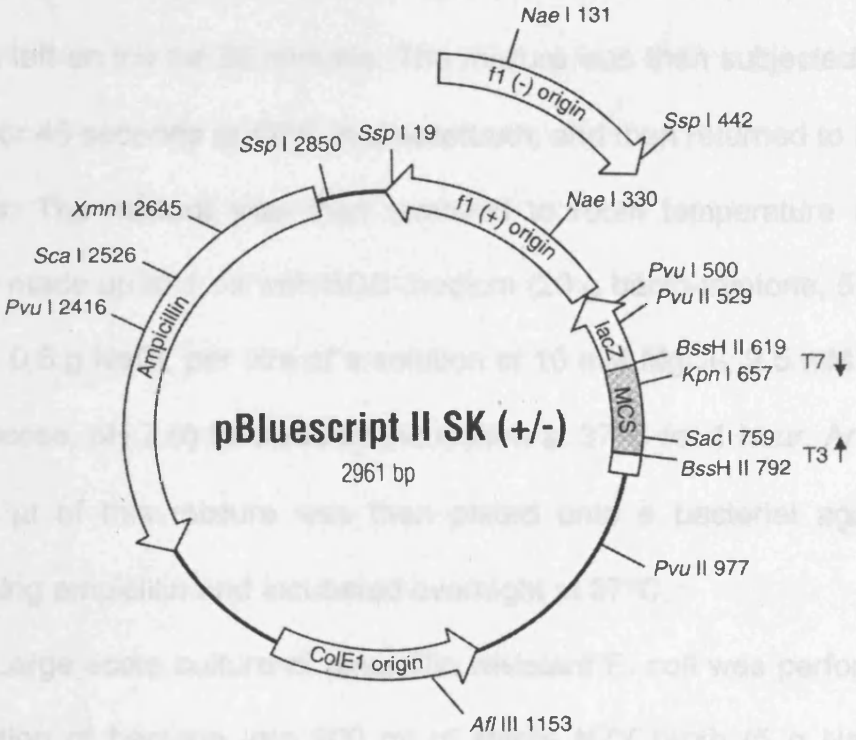
Figure 2.1
The pcDNA3 Selectable Eukaryotic Expression Vector

This vector is constructed to facilitate high level expression of recombinant proteins in mammalian cells. Promoter sequences from the immediate early gene of the human cytomegalovirus ensure high level transcription. Polyadenylation signal and transcription termination sequences from the bovine growth hormone gene enhance RNA stability. The ampicillin resistance gene allows maintenance and selection in E. coli, and the neomycin resistance gene allows selection of G418 resistant stable mammalian cell lines. A versatile multiple cloning region permits simple cloning of inserts.

pCDNA3
5.4 kb
Amp
P CMV
BGH pA
f1 ori
P SV40 ori
Neomycin
SV40 pA
ColE1
T7
Hind III
Kpn I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Apa I
Sp6

Figure 2.2
The pBluescript II SK Phagemid Vector

This vector possesses an ampicillin resistance gene for bacterial selection, and a very versatile multiple cloning region with 21 unique restriction sites. This makes the pBSII SK vector extremely useful for general cloning procedures. The multiple cloning region, together with restriction endonuclease sites is shown expanded in the lower portion of the Figure


Nae I 131
f1 (-) origin
Ssp I 442
Ssp I 2850
Ssp I 19
Nae I 330
Xmn I 2645
f1 (+) origin
Sca I 2526
Pvu I 500
Pvu II 529
Pvu I 2416
Ampicillin
lacZ
BssH II 619
T7
Kpn I 657
MCS
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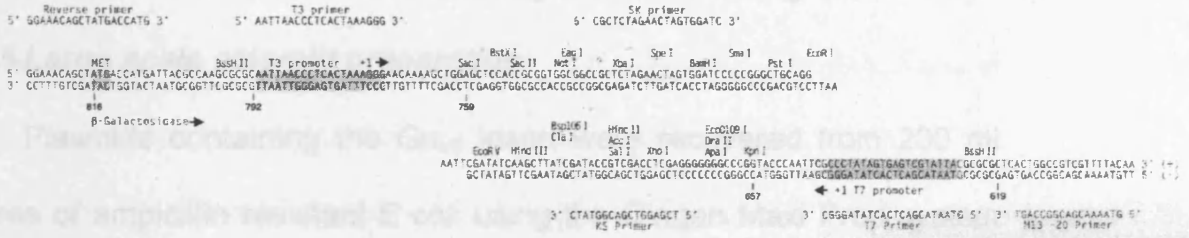
ColE1 origin
Afl III 1153


Reverse primer
5' GGAAACAGCTATGACCATG 3'
T3 primer
5' AATTAACCCTCACTAAAGGG 3'
SK primer
5' CGCTCTAGAACTAGTGGATC 3'
MET
BssH II
T3 promoter
+1
BstX I
Eag I
Spe I
Sma I
EcoR I
Sac I
Sac II
Not I
Xba I
BamH I
Pst I
5' GGAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG
3' CCTTTGTCGATACTGGTACTAATGCGGTTCGCGCGTTAATTGGGAGTGATTTCCCTTGTTTTCGACCTCGAGGTGGCGCCACCGCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAA
816
792
759
β-Galactosidase
BspM6 I
Cla I
HInc II
Acc I
Sal I
Xho I
EcoO109 I
Dra II
Apa I
Kpn I
EcoR V
HInd III
BssH II
AATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAA 3' (+)
GCTATAGTTCGAATAGCTATGGCAGCTGGAGCTCCCCCCCGGGCCATGGGTTAAGCGGGATATCACTCAGCATAATGCGCGCGAGTGACCGGCAGCAAAATGTT 5' (-)
657
+1 T7 promoter
619
3' CTATGGCAGCTGGAGCT 5'
KS Primer
3' CGGGATATCACTCAGCATAATG 5'
T7 Primer
3' TGACCGGCAGCAAAATG 5'
M13 -20 Primer

2.14.4 *Transfection of competent E. coli and bacterial culture*

The E coli strain DH5α was used for propagation of plasmids in bacterial culture. Competent cells were thawed on ice and 200 μl of cells added to a microfuge tube. Then 1 ng of plasmid DNA was added and the mixture left on ice for 30 minutes. The mixture was then subjected to heat shock for 45 seconds at 42°C in a waterbath, and then returned to ice for 2 minutes. The mixture was then removed to room temperature and the volume made up to 1 ml with SOC medium (20 g bacto-tryptone, 5 g yeast extract, 0.5 g NaCl, per litre of a solution of 10 mM $MgCl_2$, 2.5 mM KCl, 20 mM glucose, pH 7.0) followed by incubation at 37°C for 1 hour. An aliquot of 100 μl of this mixture was then plated onto a bacterial agar plate containing ampicillin and incubated overnight at 37°C.

Large scale culture of ampicillin resistant E. coli was performed by inoculation of bacteria into 200 ml of sterile NZY broth (5 g NaCl, 2 g $MgSO_4$, 5 g yeast extract, 10 g casein hydrolysate, per liter, pH 7.5) with subsequent incubation at 37°c in a shaking incubator overnight.

2.14.5 *Large scale plasmid preparation*

Plasmids containing the $G\alpha_{i-3}$ insert were recovered from 200 ml cultures of ampicillin resistant E coli using the Qiagen Maxi Prep system exactly according to the manufacturers instructions. Typically the yield from this procedure was 400 μg of plasmid DNA.

2.14.6 *Small scale plasmid preparation*

A suitable colony of bacteria was picked using a sterile toothpick and spotted onto a reference plate, and then the remainder was inoculated

into a microfuge tube containing 1.2 ml of ampicillin containing NZY broth. This procedure was followed for all the colonies to be screened and the culture tubes incubated in a shaking incubator overnight at 37°C. The next morning the tubes were centrifuged, the supernatant aspirated, and the tubes placed in a shaker. Then 200 µl of lysis mix was added and the tubes shaken for 30 seconds. The samples were then placed in a rack in a boiling water bath for 40 seconds and then microfuged for 10 minutes at 13000 rpm. The pellet was removed with a sterile toothpick, and 20 µl of 3 M Na acetate, pH 7.0, added to the supernatant followed by 250 µl of isopropanol. The samples were incubated at -70°C for 30 minutes to allow the DNA to precipitate. The samples were again microfuged for 10 minutes at 13000 rpm to pellet the DNA. The DNA pellet was washed with 70% ethanol and finally re-suspended in 50 µl of water.

2.14.7 *Plasmid DNA digests*

DNA digests were performed in a final volume of either 10 µl or 20 µl at 37°C for 1 hour. For those digestions using *Hind* III, *EcoR* I and *Xho* I the reaction buffer was composed of 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl. Reaction products were visualised by running on 1% agarose gels containing 0.5 µg/ml ethidium bromide, followed by ultraviolet illumination and photographing.

2.14.8 *Purification of digested DNA fragments*

Product of the appropriate DNA digestion reaction were run on a 1% agarose gel containing 0.5 µg/ml ethidium bromide until the band of interest was well separated from others. The band was then excised from

the gel in as small a piece of agarose as possible. A short length of visking tubing was washed in 0.1 x TAE buffer (1 x TAE is 40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and the piece of agarose gel inserted into the tubing together with 3 ml of 0.1 xTAE buffer. The tubing was then placed in a gel tank containing 0.1 xTAE buffer and the DNA eluted from the gel by the application of 200 V for one hour. A UV transilluminator was used to confirm elution of DNA from the gel slice which was then discarded. The 3 ml of buffer containing the eluted DNA was collected and the DNA purified using the Wizard Miniprep DNA purification system (Promega, Southampton, UK) according to the manufacturers instructions. The DNA was precipitated in 99% ethanol, washed, dried, re-dissolved in sterile distilled water and stored at -20°C.

2.14.9 ***Ligation of purified DNA fragments***

The ligation of DNA fragments was performed in a final volume of 20 µl in a buffer composed of (final concentrations) 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 5% (w/v) polyethylene glycol-8000. A quantity of 30 ng of appropriately digested plasmid DNA was mixed with $G\alpha_{i-3}$ cDNA insert using a molar ratio of 1:3, plasmid DNA:insert DNA. The ligation reaction was catalysed by addition of 1 unit of T4 DNA ligase, followed by incubation at 16°C for 4 hours. The products of these ligation reactions were then used for the transfection of competent DH5α E. coli as described above.

2.14.10 ***Transfection of Plasmid/$G\alpha_{i-3}$ Constructs into OK Cells***

Transfections were performed using the calcium phosphate precipitation method. 10 μg of supercoiled plasmid DNA was added to 0.1 xTAE buffer (pH 8.0) to make a final volume of 440 μl. To this solution was added 500 μl of 2x HBS (280 mM NaCl, 10mM KCl, 1.5 mM Na_2HPO_4, 11 mM glucose, 50 mM HEPES, pH 7.05). Then 62 μl of 2 M $CaCl_2$ was added whilst bubbling gently with air to enable mixing. This mixture was incubated at room temperature for 30 minutes. This mixture was then added directly to a 10 cm petri dish containing exponentially growing OK cells, ~ 50% confluent, in 10 mls of DMEM F-12 medium. These cells were then incubated as normal for a further 24 hours, then the medium/DNA mixture was removed and then replaced with 10 mls of fresh DMEM F-12 followed by incubation for a further 24 hours. Transfected cells were then split using trypsin/EDTA and re-seeded into DMEM F-12 containing 300 μg/ml G418 to commence selection.

Individual resistant colonies were carefully picked after approximately 5 weeks in G418 selection medium, amplified separately, and then screened for expression of the transfected gene.

2.14.11 ***Screening of transfected OK cells***

OK cells resistant to G418 were screened for expression of $G\alpha_{i-3}$ protein using anti-$G\alpha_{i-3}$ antibodies. Resistant cells were amplified progressively from individual discrete colonies in 10 cm petri dishes, until confluent in 80 cm^2 culture flasks. G418 was retained in the medium throughout this period. Membranes were then prepared from these cells and subjected to immunoblotting as described above.

2.14.12 *Use of transfected cells*

Cell lines stably transfected with either the pcDNA3/$G\alpha_{i-3}$ construct or pcDNA3 plasmid alone were established. These cell lines were then used for albumin binding and uptake experiments exactly as described above.

2.15 Examination of the Potential Role of Protein Kinase C in the Regulation of Albumin Endocytosis

The potentially important role of PKC in the regulation of endocytic events was discussed earlier. In order to assess the effect of PKC activity on [^{125}I]-albumin endocytosis in wild type OK cells phorbol esters were used to pre-treat the cells in culture. Phorbol esters are tumour promoting agents which exert their cellular effects by stimulating PKC in a manner similar to diacylglycerol. The phorbol esters have been very widely used to stimulate PKC activity in cell culture systems (Ryves *et al* 1991, Nishizuka 1992, Hug and Sarre 1993, Mosior and Newton 1995).

Wild type OK cells were grown to confluence in 6 well plates. On the morning of the experiment cells were placed in serum free DMEM F12 medium for 2 hours at 37°C as already described. Following this the cells were treated with 1 μM phorbol 12, 13 dibutyrate (PDBU) in serum free DMEM F12 for 10 minutes at 37°C. [^{125}I]-albumin uptake was then started by incubating the cells with various concentrations of [^{125}I]-albumin diluted in DMEM F12 containing 1 μM PDBU. Uptake was allowed to proceed for 15 minutes before being stopped with ice cold PBS as previously described.

2.16 Examination of the Potential Role of Phospholipase D in the Regulation of Albumin Endocytosis

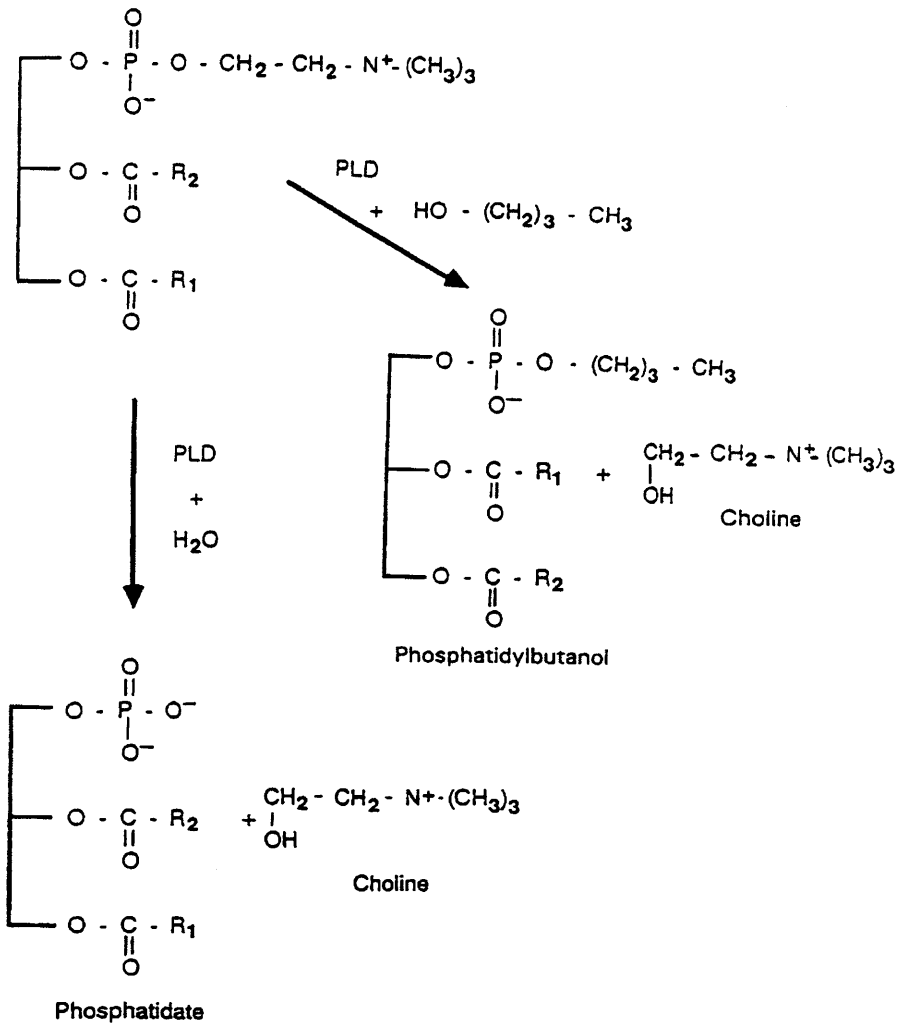
In order to modulate the activity of PLD, and thus assess it's influence on the receptor mediated endocytosis of albumin, advantage was taken of the transphosphatidylation reaction. This approach uses a primary alcohol, in this case butanol, to divert the action of PLD from the production of phosphatidic acid into the production of phosphatidylbutanol, thus effecting functional inhibition of the enzyme activity (Boarder and Purkiss 1993, Ktistakis *et al* 1996) (Figure 2.3).

Confluent OK cells were grown in 6 well plates and quiesced for 2 hours in serum free DMEM F12 on the morning of the experiment as previously described. This media was then replaced with serum free DMEM F12 containing various concentrations of butanol and the cells incubated for 10 minutes at 37°C. This media was then removed and albumin uptake was then initiated by the addition of further butanol containing serum free DMEM F12 together with 150 mg/L [^{125}I]-albumin. Uptake was allowed to continue for 15 minutes at 37°C. Cells were then processed as described previously.

Figure 2.3
The Action of Phospholipase D

The action of phospholipase D on it's dominant substrate, the phospholipid phosphatidylcholine (ester linked acyl or ether linked alkyl groups are designated as R_1 and R_2), leads to the formation of a free phosphatidate moiety, choline and H^+. However in the presence of an alcohol such as butanol ($CH_3(CH_2)_3OH$) the relevant phosphatidylalcohol (phosphatidylbutanol) is formed by a transphosphatidylation reaction. With millimolar concentrations of alcohols such as butanol essentially complete diversion of the reaction to transphosphatidylation can occur. The enzyme is thus in effect inhibited because the active signalling product phosphatidic acid is no longer formed by phospholipase D activity.

The Action of Phospholipase D



Chapter 3

Results 1

CHARACTERISATION OF THE ALBUMIN BINDING SITES IN OK CELLS

3.1 Preliminary Experiments

3.1.1 *Verification of washing protocol*

In order to confirm that the washing protocol for cell monolayers after [^{125}I]-albumin binding or uptake had been performed was adequate to remove any free or unbound radiolabel, aliquots of PBS used for each of 5 consecutive washes were counted after aspiration from the cell monolayer. The radioactivity in each of these aliquots was compared to that in the [^{125}I]-albumin containing incubation solution (wash 0) and the results are shown in Figure 3.1. These results clearly demonstrate that washing the cell monolayers three times with 2 ml PBS is sufficient to remove all free radioactivity from the wells, such that radioactivity in subsequent washes is reduced to background levels. Therefore any remaining radioactivity in the culture dishes must represent [^{125}I]-albumin either bound to the cell surface or taken up into the cells.

3.1.2 *Choice of cell type*

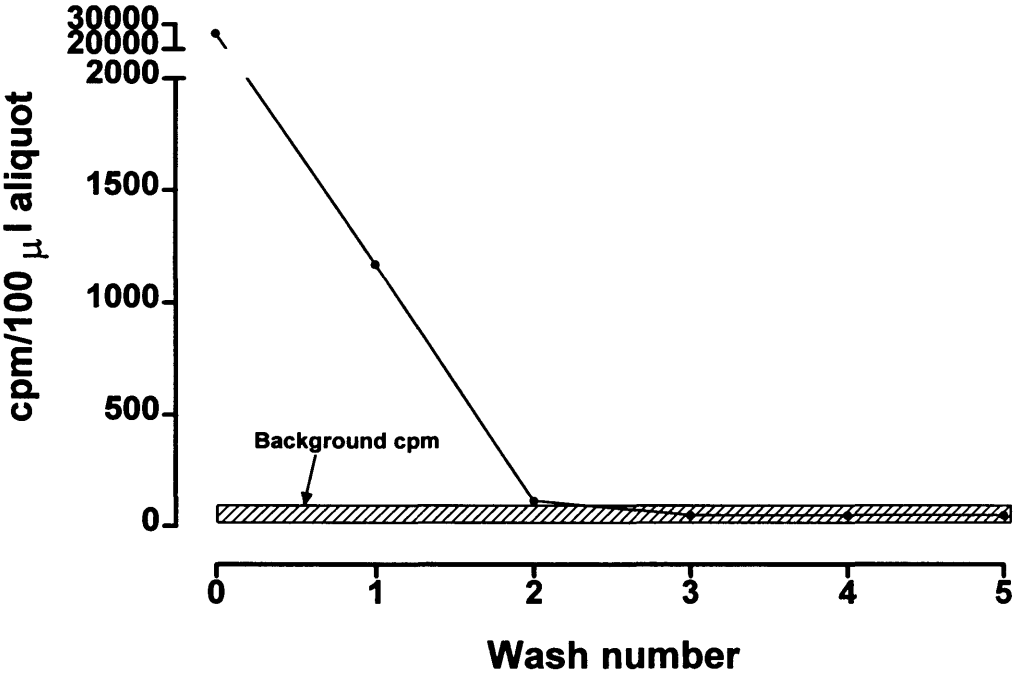


Figure 3.1
Verification of OK Cell Monolayer Washing Protocol

OK cell monolayers were grown to confluence in 6 well plates and [^{125}I]-albumin uptake allowed to continue for 15 minutes using various concentrations of [^{125}I]-albumin. Following the uptake experiment monolayers were washed 3 times with 2 ml of ice cold PBS. To ensure that this washing protocol wash sufficient for the removal of any free radioactivity in the wells a 100 µl aliquot of the initial incubation medium was counted for radioactivity, and this was then compared to the radioactivity in an equal aliquot of PBS derived from each of the washing steps. After 2 washes radioactivity in the PBS washing solution has fallen to background levels indicating that free radioactivity has been washed away.

These preliminary experiments were designed to determine which tubular epithelial cell type would be the most suitable for the planned studies. Therefore [^{125}I]-albumin saturation uptake experiments were performed in both confluent $LLCPK_1$ cells and OK cells at a time point of 15 minutes at 37°C. The results were corrected for protein concentration and compared. The results shown in Figure 3.2 demonstrate a qualitatively similar pattern of saturable albumin binding to both of these cell types, compatible with a receptor mediated process. However, quantitatively albumin uptake into the OK cells was greater than into the $LLCPK_1$ cells. This difference is accounted for in the main by a much larger V_{max} for the uptake process in the OK cells (354.4 ± 67.7 ng albumin/mg protein, n = 2) compared to the $LLCPK_1$ cells (86.6 ± 20.7 ng albumin/mg protein, n = 2).

The results of these preliminary experiments therefore suggested that albumin uptake was a considerably more prominent function of OK cells compared to $LLCPK_1$ cells. On the basis of these observations therefore it was decided that OK cells were the most appropriate proximal tubular cell model system for the *in vitro* study of albumin binding and endocytosis. All subsequent experimental results depict observations in OK cells.

3.2 Characterisation and Identification of Albumin Binding Proteins in OK Cells

3.2.1 *Time course of [^{125}I]-albumin binding to OK cells*

All [^{125}I]-albumin binding experiments were performed at 4°C to prevent internalisation. In order to be able to determine the equilibrium

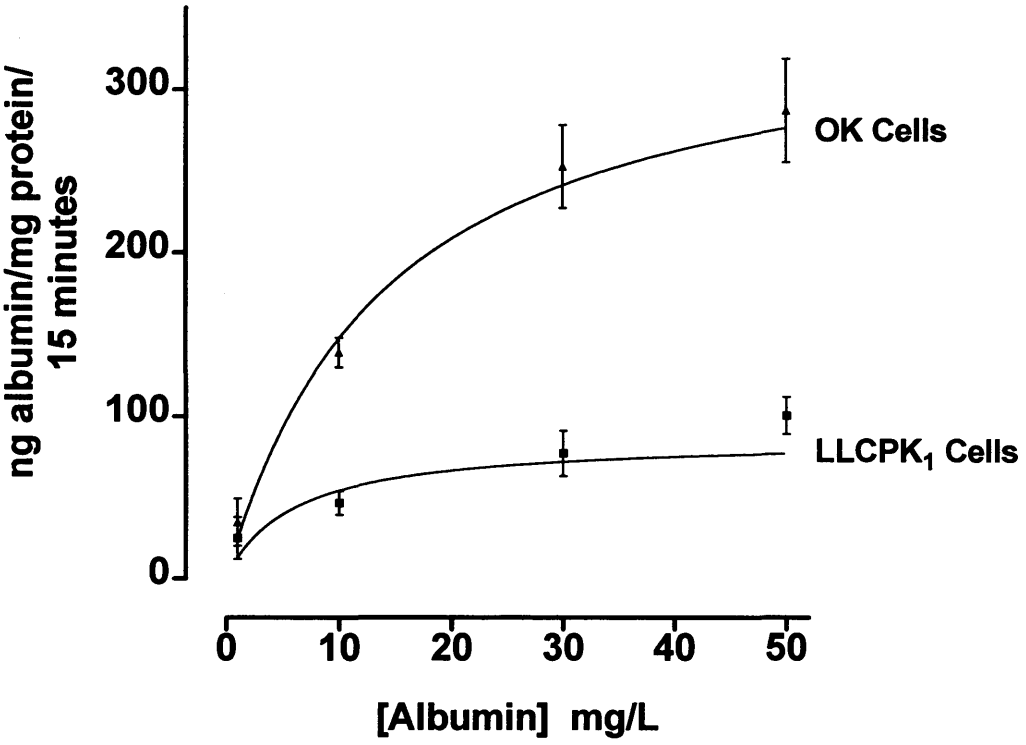


Figure 3.2
Comparison of Albumin Endocytosis in Two Different Proximal Tubule Derived Cell Lines

In order to determine the most suitable cell line to use for the bulk of the albumin binding and endocytosis studies, [^{125}I]-albumin endocytosis was measured in both confluent OK and $LLCPK_1$ cells grown in 6 well plates under identical conditions and the results compared. Uptake was allowed to proceed at 37°C for 15 minutes.Values represent means ± SE of n = 3 experiments performed in duplicate. Values representing binding have been subtracted.

dissociation constant (K_D) of an albumin binding site(s) it was initially necessary to ascertain when the binding of albumin to OK cells reached equilibrium. Thus the time course of [^{125}I]-albumin binding to OK cells was measured. The time course of 50 mg/L [^{125}I]-albumin binding to OK cells at 4°C is depicted in Figure 3.3. It demonstrates that although binding proceeds rapidly, binding equilibrium is not reached until several hours of binding had elapsed.

3.2.2 *Determination of K_D for albumin binding and receptor number*

Based on the results of the time course binding experiments, saturation binding of albumin was examined by incubating cell monolayers with [^{125}I]-albumin overnight at 4°C when binding was at equilibrium.

Total binding of [^{125}I]-albumin is made up of two components, specific and non-specific binding. Non-specific binding was defined in the presence of a large excess of unlabelled albumin, demonstrated a linear relationship with the concentration of [^{125}I]-albumin in the incubating solution, and comprised approximately 25% of the total binding. When the values for non-specific binding are subtracted from total [^{125}I]-albumin binding this gives the value for specific binding. These values for total, non-specific and specific binding of [^{125}I]-albumin to OK cells are shown in Figure 3.4.

The process of [^{125}I]-albumin binding to OK cells at 4°C demonstrates saturation kinetics consistent with a receptor mediated process. Saturation binding curves of [^{125}I]-albumin to OK cells at 4°C after overnight incubation were constructed, and the data analysed by non-

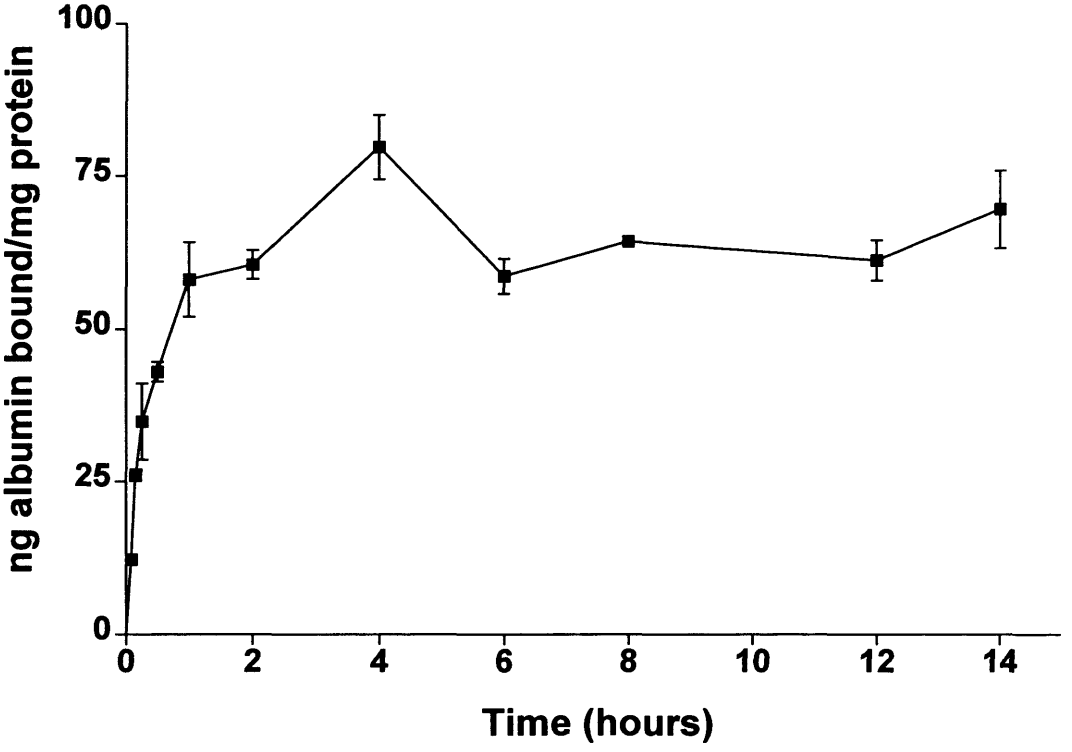


Figure 3.3
Time Course of Specific [^{125}I]-Albumin Binding to Wild Type OK Cells

OK cells were grown to confluence in 6 well plates. [^{125}I]-albumin binding was measured at 4°C for various times. An [^{125}I]-albumin concentration of 50 mg/L was used in all experiments. Each point represents mean ± SE, of n ≥ 3 experiments performed in duplicate. Non-specific binding values have been subtracted.

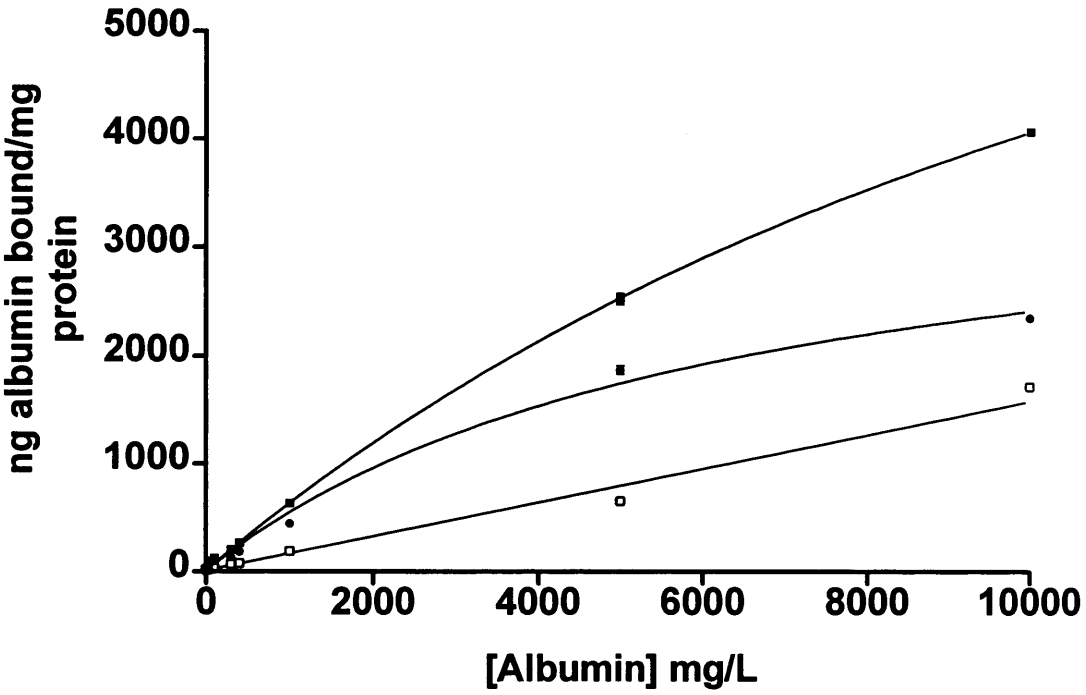


Figure 3.4
[^{125}I]-Albumin Binding to OK Cell Monolayers

Wild type OK cells were grown to confluence in 6 well plates. Cells were incubated with various concentrations of [^{125}I]-albumin at 4°C overnight. Binding can be separated into total (■), non-specific (□), and specific (●) binding components. Non-specific binding was defined in the presence of a large excess of unlabelled albumin. Values represent means ± SE derived fro a singlr representative experiment performed in duplicate. Specific binding is determined by subtracting non-specific from total binding. Non-specific binding demonstrates a linear relationship with increasing incubated [^{125}I]-albumin concentration, whereas specific binding is saturable.

linear curve fitting to a two-binding site model for the calculation of K_D and B_{max}. A representative experiment is shown in Figure 3.5, together with a Scatchard plot of the transformed data (Figure 3.6).

In all the experiments performed two components of specific albumin binding are apparent, an early component which saturates at low albumin concentrations, and a second components which saturates at much higher concentrations of albumin. Analysis of these data by non-linear curve fitting confirms the presence of two albumin binding sites, one with high affinity and low capacity, and one with low affinity and high capacity. The K_D value for the high affinity site is 154.8 ± 7 mg/L, with a B_{max} of 228.4 ± 9.1 ng/mg protein (n = 3 experiments), and the K_D value for the low affinity site is 8300.0 ± 1000 mg/L, with B_{max} 4630.0 ± 717 ng/mg protein (n = 3 experiments). The Scatchard plot of the transformed data clearly demonstrates the existence of these two binding sites for albumin.

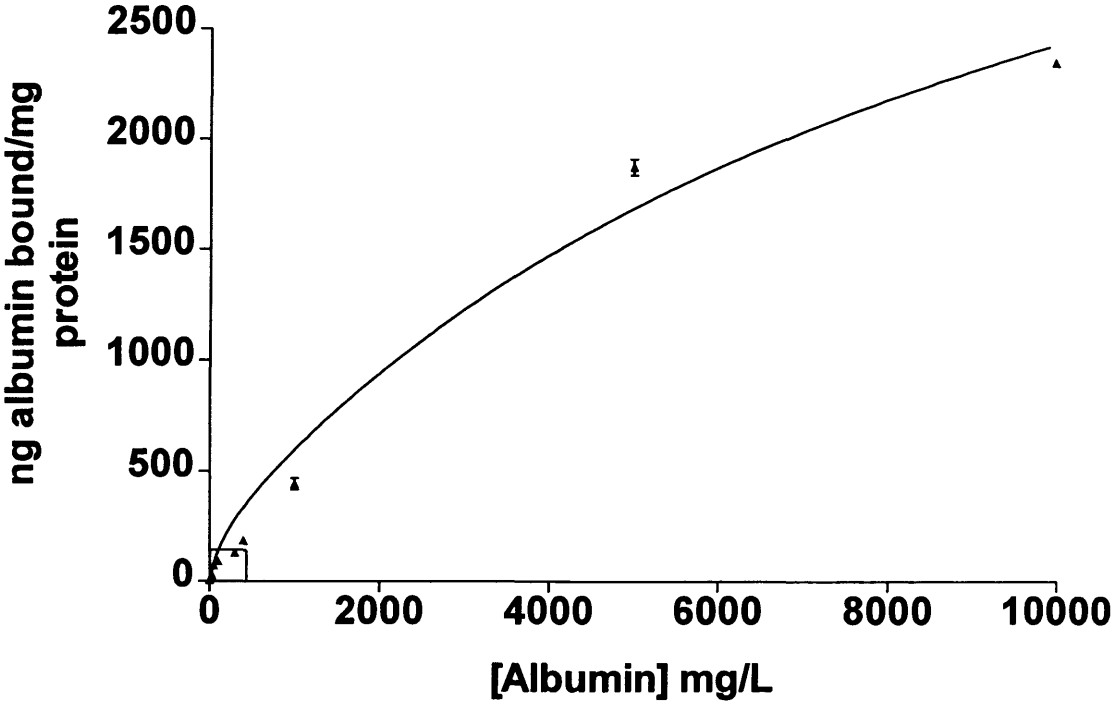
3.2.3 *Lectin competition of [^{125}I]-albumin binding*

After determining in the saturation binding experiments detailed above that the K_D of the higher affinity binding site for albumin was of the order of 150 mg/L, the lectin competition experiments were performed using an [^{125}I]-albumin concentration of 150 mg/L in the incubating solution.

When OK cells were pre-incubated with lectins several alterations in [^{125}I]-albumin were observed. No inhibition of [^{125}I]-albumin binding was seen with the lectins Con A, BS II, AH, or LP with incubated lectin concentrations of up to 100 μg/ml. However when cell monolayers were

Figure 3.5
Saturation Binding of [^{125}I]-Albumin to OK Cells

Wild type OK cells were grown to confluence in 6 well plates and [^{125}I]-albumin binding was measured at 4°C by incubating cell monolayers with various concentrations of [^{125}I]-albumin overnight. The upper graph represents binding across the entire concentration range of [^{125}I]-albumin used in the experiments. The lower graph represents binding over the lower concentration range of [^{125}I]-albumin used in the experiments (boxed on upper graph). Data represent means ± SE derived from one representative experiment performed in duplicate. Values for non-specific, binding measured in the presence of a large excess of unlabelled albumin, have been subtracted. These data were analysed by non-linear curve fitting using GraphPad Prism computer software. This analysis revealed the presence of two binding sites for albumin. The K_D value for the higher affinity site calculated from this experimental data is 156.1 mg/L, the K_D for the lower affinity site is 10200 mg/L.


2500
2000
1500
1000
500
0
ng albumin bound/mg protein
0
2000
4000
6000
8000
10000
[Albumin] mg/L

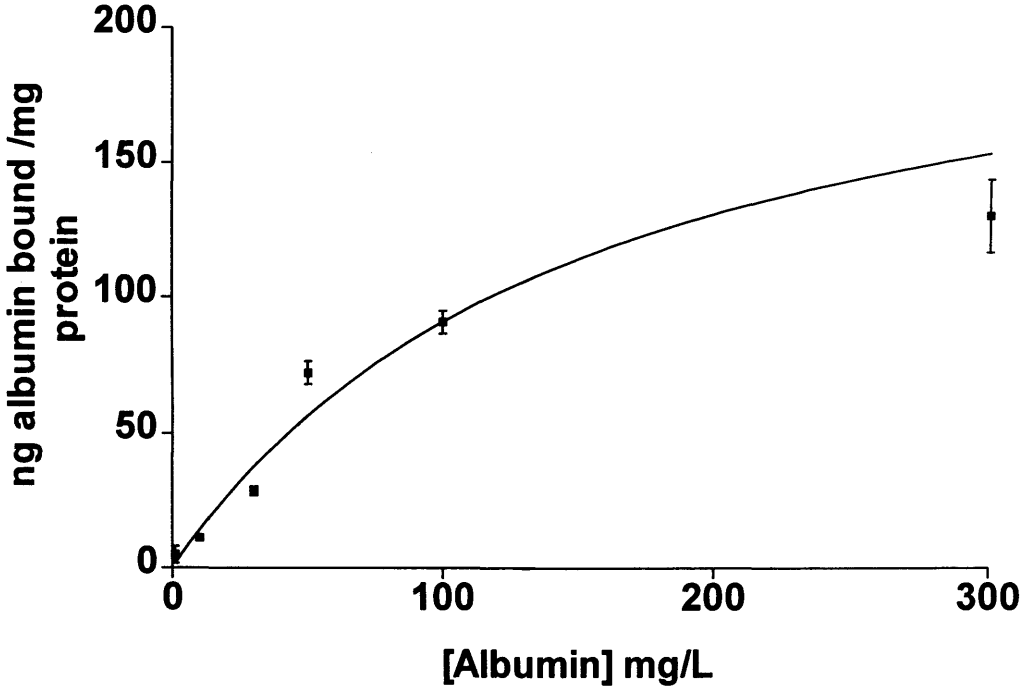
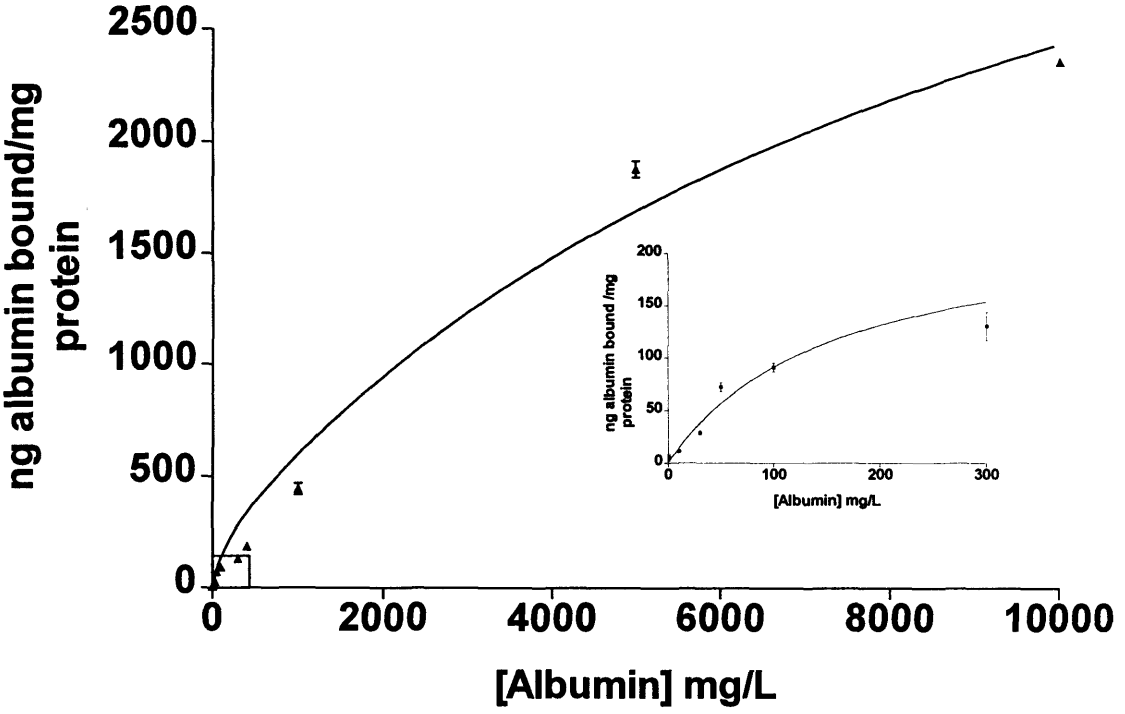
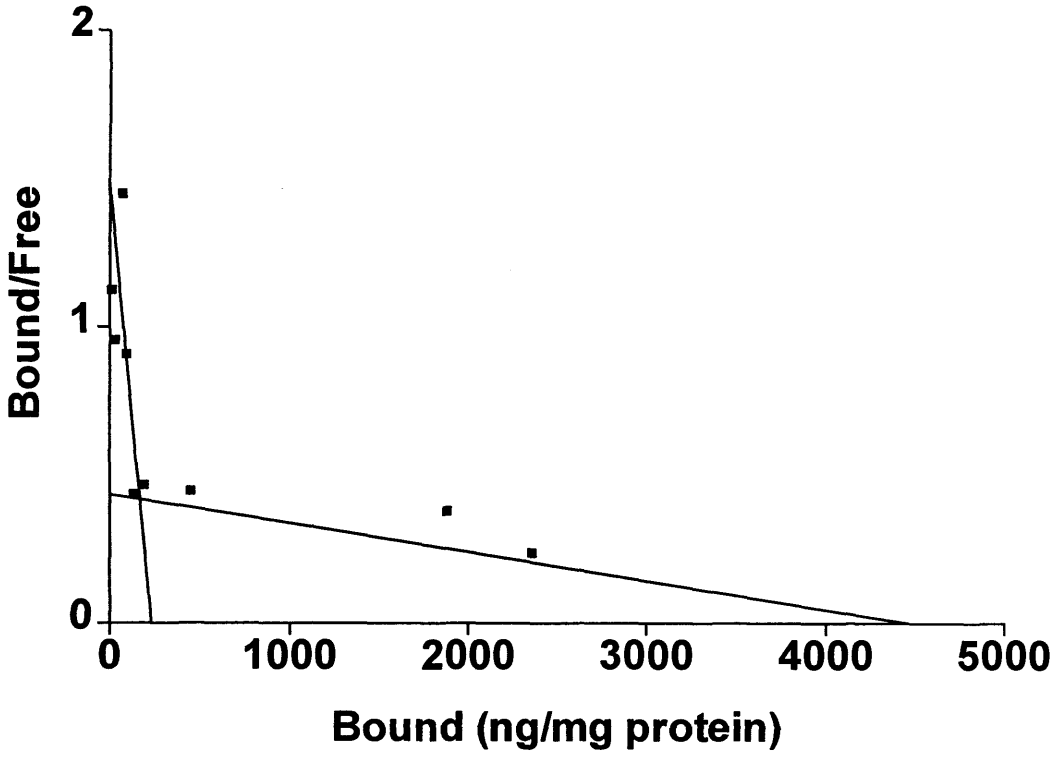

200
150
100
50
0
ng albumin bound /mg protein
0
100
200
300
[Albumin] mg/L

Figure 3.6
Saturation Binding of [^{125}I]-Albumin to OK Cells

Wild type OK cells were grown to confluence in 6 well plates and [^{125}I]-albumin binding was measured at 4°C by incubating cell monolayers with various concentrations of [^{125}I]-albumin overnight. The upper graph and inset depict the saturation binding curves shown in the previous figure. In the lower graph this data has been transformed and displayed for clarity as a Scatchard plot using GraphPad Prism computer software. This transformation clearly demonstrates the presence of two albumin binding sites, the higher affinity site being represented by the steeper line and the lower affinity site by the less steep line. However this type of data transformation was not used for analysis of data and calculation of binding constants.


ng albumin bound/mg protein
2500
2000
1500
1000
500
0
0
2000
4000
6000
8000
10000
[Albumin] mg/L
ng albumin bound /mg protein
200
150
100
50
0
0
100
200
300
[Albumin] mg/L


Bound/Free
2
1
0
0
1000
2000
3000
4000
5000
Bound (ng/mg protein)

pre-incubated with the lectins of UE or GM, specific [^{125}I]-albumin binding was significantly inhibited (Fig. 3.7). The observed inhibition of [^{125}I]-albumin binding by UE and GM was concentration dependent, approximately 50% of specific [^{125}I]-albumin binding being inhibited at a lectin pre-incubation concentration of 50 μg/ml. Furthermore these lectins, UE and GM, demonstrated very similar concentration dependency curves of inhibition of [^{125}I]-albumin binding to OK cell monolayers at 4°C (Figure 3.8), each with an IC_{50} of approximately 50 μg/ml. Other lectins demonstrated no inhibition of albumin binding with increasing concentration.

The inhibition of [^{125}I]-albumin binding observed with UE and GM was entirely overcome by pre-incubation of the lectin with its cognate sugar hapten (Figure 3.7). This indicates that the observed binding inhibition was due to a specific lectin/sugar interaction rather than simple competition between the two proteins, lectin and albumin, for a single binding site.

3.2.4 *Identification of [^{125}I]-albumin binding proteins by ligand blotting*

In order to identify individual albumin binding proteins in OK cell membranes the technique of ligand blotting was employed, using as a ligand the same [^{125}I]-albumin preparation used in binding experiments to cell monolayers. Initially several different methods of sample preparation were employed utilising both reducing and non-reducing conditions with or without boiling of the protein samples prior to loading onto polyacrylamide

Figure 3.7
Lectin-Evoked Inhibition of [^{125}I]-Albumin Binding to OK Cell Monolayers

The effect of pre-incubation of wild type OK cell monolayers with 50 μg/ml lectin on subsequent binding of 150 mg/L [^{125}I]-albumin was measured at 4°C. (C Control representing OK cell monolayers incubated with [^{125}I]-albumin alone with no lectin pre-incubation. Other abbreviations in text). Where significant inhibition of binding was observed hatched bars represent binding of [^{125}I]-albumin after pre-incubation of OK cells with the same lectin pre-incubated with it's cognate sugar hapten. Bars represent means ± SE; $n \geq 4$ experiments performed in duplicate. Significant differences are shown: * $p = 0.003$, ** $p = 0.0038$.

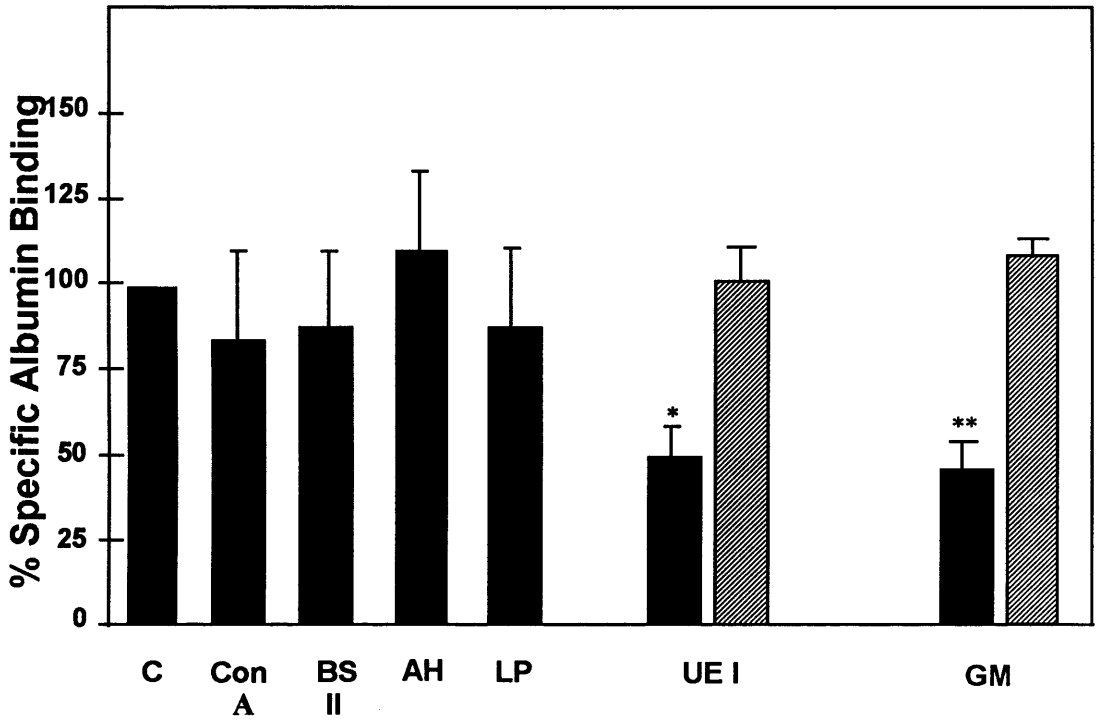

% Specific Albumin Binding
150
125
100
75
50
25
0
*
**
C
Con A
BS II
AH
LP
UE I
GM

Figure 3.8
Concentration Dependency of Lectin Inhibition of [^{125}I]-Albumin Binding to OK Cells

Wild type OK cells were grown to confluence in 6 well plates. Binding of 150 mg/L [^{125}I]-albumin was measured at 4°C in the presence of various lectins at various concentrations. The lectins Glycine max and Ulex europaeus I demonstrate dose dependent inhibition of specific [^{125}I]-albumin binding. Other lectins show no inhibition of binding. Values represent means ± SE; n ≥ 3 experiments performed in duplicate.

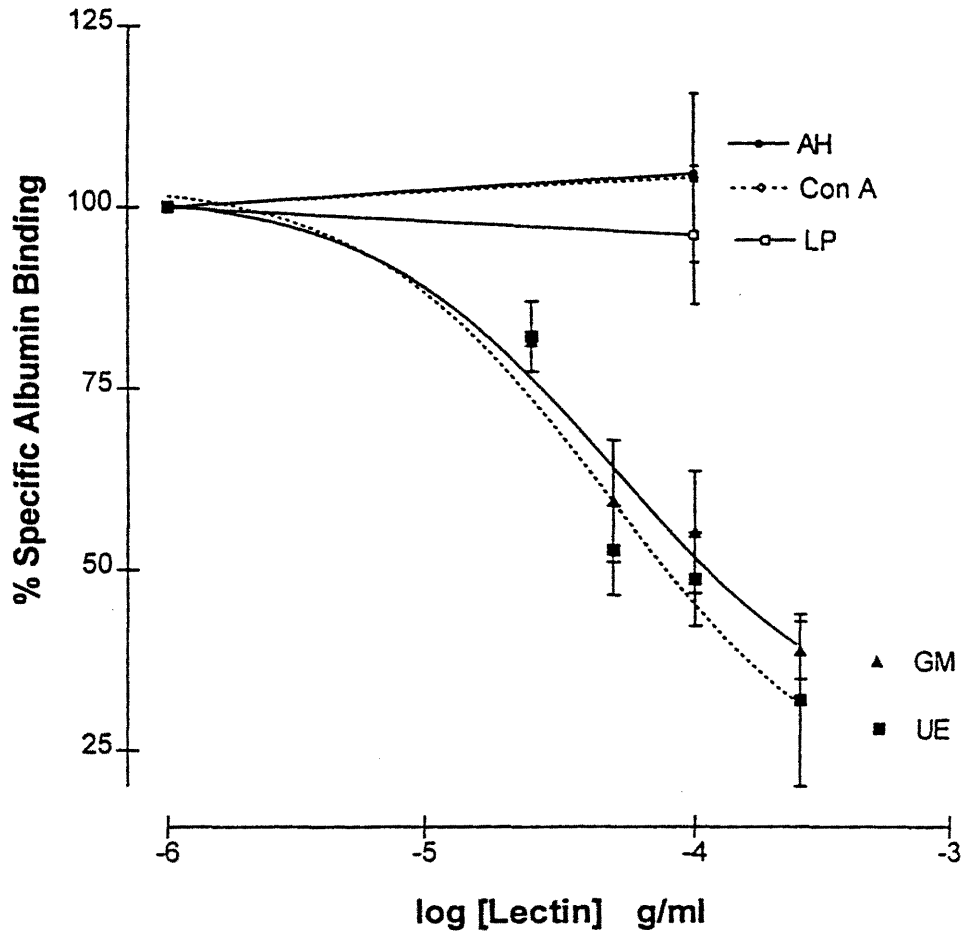

125
100
75
50
25
% Specific Albumin Binding
AH
Con A
LP
GM
UE
-6
-5
-4
-3
log [Lectin] g/ml

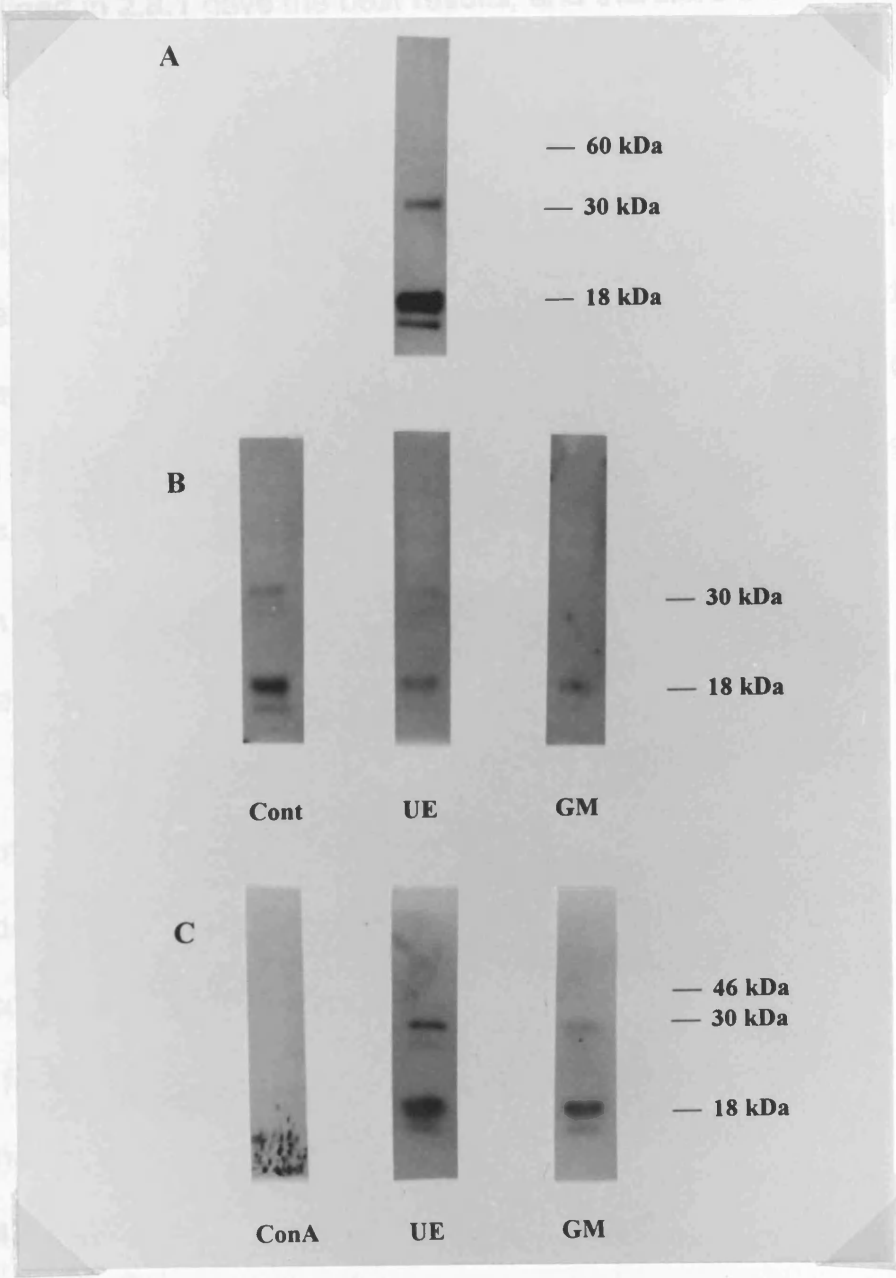
Figure 3.9

Autoradiographs of [^{125}I]-Albumin Ligand Blots, Lectin/[^{125}I]-Albumin Competition Blots, and Lectin Blots of OK Cell Membranes

A. [^{125}I]-albumin ligand blot performed under control conditions. A nitrocelluose strip carrying separated OK cell membrane proteins has been incubated with [^{125}I]-albumin, washed and autoradiographed. Several bands are observed to bind [^{125}I]-albumin, and represent albumin binding proteins.

B. [^{125}I]-albumin ligand blot performed under control conditions with [^{125}I]-albumin alone (cont), or in the presence of the indicated competing lectins. Albumin binding is reduced only in the presence of the lectins GM and UE.

C. Autoradiograph of peroxidase labelled lectin binding to OK cell membrane proteins on nitrocellulose, developed with a chemiluminescent detection system. The lectins GM and UE bind proteins of the same molecular weight as [^{125}I]-albumin. The lectin Con A binds only to a faint band at 46 kDa.
All the molecular weights marked were calculated according to the mobility of co-migrating molecular weight standards.


A
— 60 kDa
— 30 kDa
— 18 kDa
B
— 30 kDa
— 18 kDa
Cont
UE
GM
C
— 46 kDa
— 30 kDa
— 18 kDa
ConA
UE
GM

gels. Furthermore a variety of blocking agents were employed in order to optimise the specific binding of [^{125}I]-albumin whilst reducing non-specific background binding. These preliminary experiments demonstrated that the conditions outlined in **2.8.1** gave the best results, and therefore the results depicted in this thesis were obtained using this methodology.

A representative autoradiograph of an [^{125}I]-albumin ligand blot is depicted in Figure 3.9.A. A number of radiolabelled bands are observed which represent proteins in the OK cell membranes which are able to bind albumin. Three major bands of [^{125}I]-albumin binding are observed at 14 kDa, 18 kDa and 30 kDa, together with a minor band at 60 kDa. The binding of [^{125}I]-albumin to these protein bands is completely abolished by co-incubation with a large excess of unlabelled human serum albumin.

3.2.5 *Concentration dependency of [^{125}I]-albumin binding in ligand blots.*

The intensity of labelling of the 18 kDa and 30 kDa albumin binding protein bands was measured by densitometry after ligand blotting with increasing concentrations of [^{125}I]-albumin. Data were analysed by non-linear curve fitting. The binding of [^{125}I]-albumin to both of these bands was saturable and concentration dependent. The K_D for the 30 kDa site was 113.2 ± 28.43 mg/L (Figure 3.10). The K_D for the 18 kDa band was 123.9 ± 32.3 mg/L, n = 3 (Figure 3.11). These figures are broadly in agreement with the values for binding of [^{125}I]-albumin to the higher affinity sites in the OK cell monolayers. The implication of these findings is that the 18 kDa protein may be a shortened or truncated form of the 30 kDa protein, and

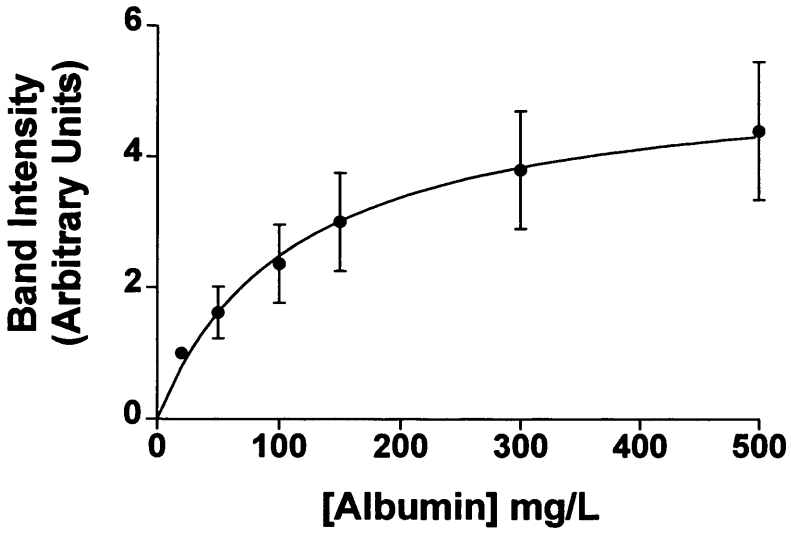


Figure 3.10
Concentration Dependence of [^{125}I]-Albumin Binding to the 30 kDa Band on OK Cell Membrane Ligand Blots

OK cell membrane proteins were separated by PAGE and transferred to nitrocellulose membranes. These nitrocellulose membranes were then incubated with the indicated concentrations of [^{125}I]-albumin, washed and autoradiographed. Labelled bands were quantified by laser densitometry. The densitometry value for the lowest incubated concentration of [^{125}I]-albumin was arbitrarily assigned the value 1, and all other densitometry readings were assigned values according ot their fold increase in labelling over that seen with the lowest [^{125}I]-albumin concentration. Values represent means ± SE; n = 3 separate blots.

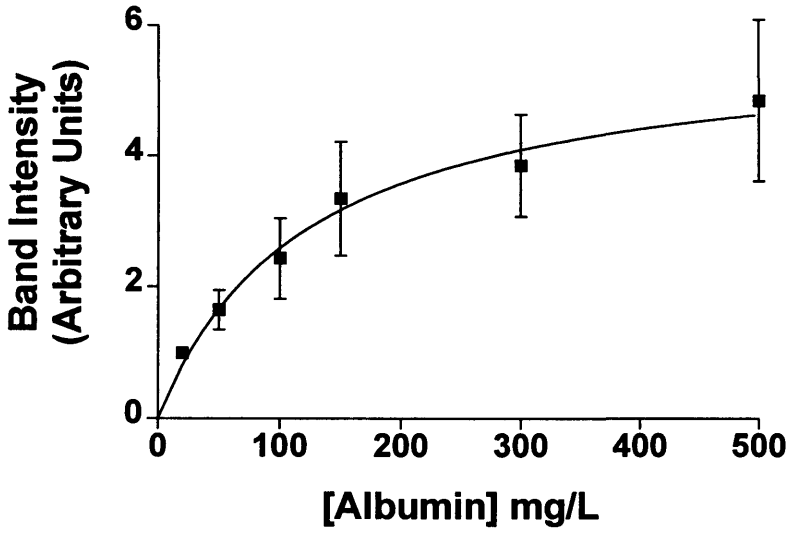


Figure 3.11
Concentration Dependence of [^{125}I]-Albumin Binding to the 18 kDa Band on OK Cell Membrane Ligand Blots

OK cell membrane proteins were separated by PAGE and transferred to nitrocellulose membranes. These nitrocellulose membranes were then incubated with the indicated concentrations of [^{125}I]-albumin, washed and autoradiographed. Labelled bands were quantified by laser densitometry. The densitometry value for the lowest incubated concentration of [^{125}I]-albumin was arbitrarily assigned the value 1, and all other densitometry readings were assigned values according ot their fold increase in labelling over that seen with the lowest [^{125}I]-albumin concentration. Values represent means ± SE; n = 3 separate blots.

furthermore this protein(s) is likely to represent the higher affinity binding site for albumin identified in intact cells.

3.2.6 *Lectin/[^{125}I]-albumin ligand blot competition experiments*

When ligand blotting experiments were performed with the lectins GM and UEA I present in the incubating solutions, the binding of [^{125}I]-albumin to the protein bands shown in Figure 3.9.A was markedly inhibited as compared to controls incubated with the [^{125}I]-albumin alone (Figure 3.9.B). Furthermore, when an irrelevant lectin, which failed to inhibit binding in OK cell monolayers, was employed no inhibition of [^{125}I]-albumin binding to any of the labelled bands was observed.

3.2.7 *Lectin blotting of OK cell membrane proteins*

The lectins GM and UEA I were also found to bind to OK cell proteins with precisely the same molecular weights in ligand blots as did the [^{125}I]-albumin (Figure 3.9.C). The lectin Con A however bound to none of these protein bands, but faintly labelled a protein band at 46 kDa which did not bind [^{125}I]-albumin. This lectin was also unable to block the binding of albumin to OK cell monolayers (Figure 3.7).

3.2.8 *Specificity of albumin binding sites*

In order to determine the specificity of the albumin binding sites in OK cell monolayers a number of potential competitors were used in an attempt to displace the binding of [^{125}I]-albumin. The competitors used in these experiments fall into two broad classes. The first is a group of proteins which are found in normal or inflamed urine, and may be in a natural position to compete for a protein receptor in the proximal tubule

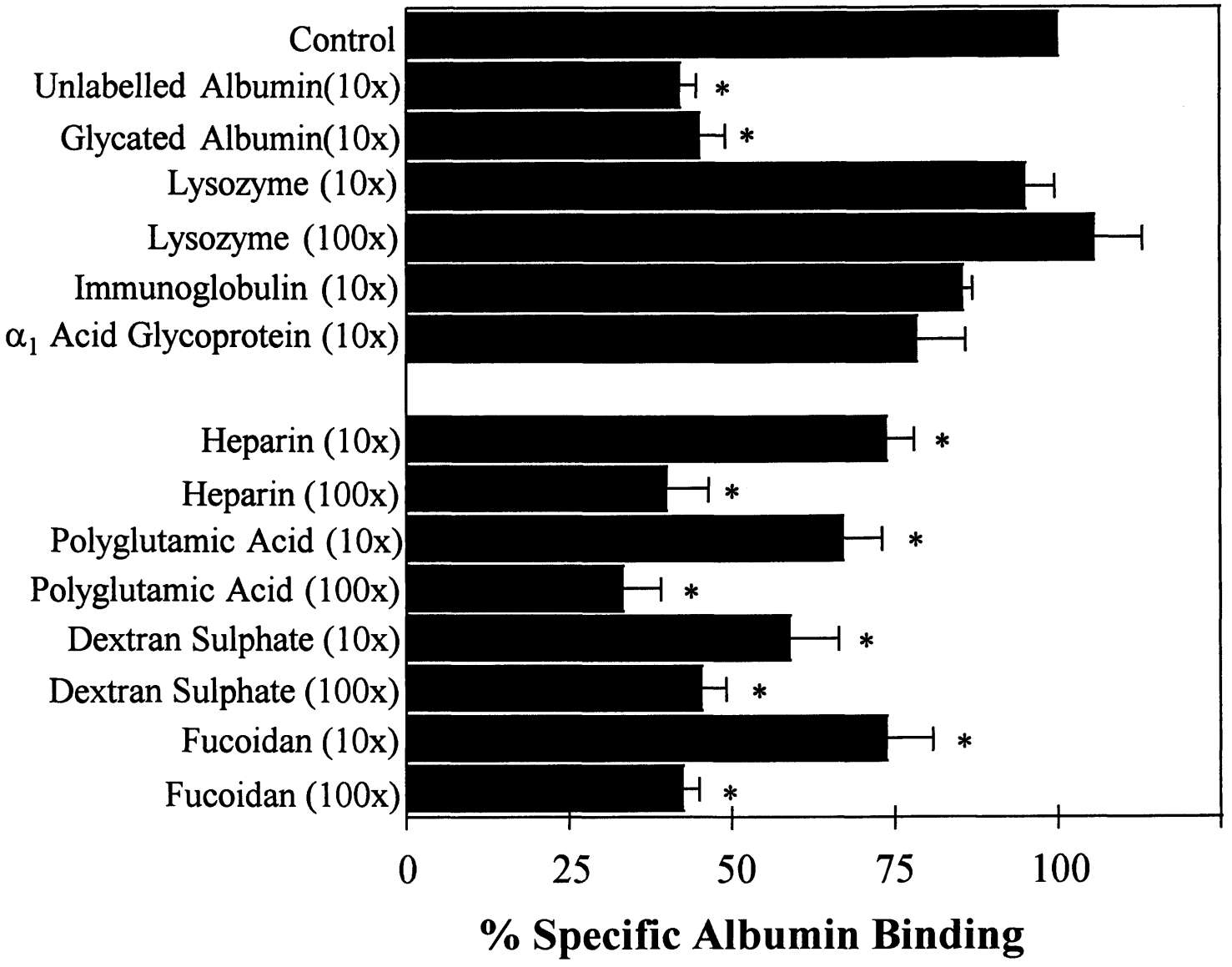
epithelium. The second group comprises a number of polyanionic macromolecules which have been previously documented to inhibit the binding of albumin and other ligands to scavenger receptors. In all these experiments the radiolabelled albumin was incubated with the cells at a concentration of 150 mg/L, the K_D of the high affinity binding site detected in the saturation binding experiments described above. Thus in these experiments the majority of albumin bound to the monolayers would be bound to the high affinity site. All the potential competitors were used at a 10 fold molar excess (10x) over the concentration of the radiolabel, and in some experiments a 100 fold molar excess (100x) of competitor was employed (Fig. 3.12).

When unlabelled human serum albumin was used as a competitor at 10x approximately 60% of the binding of the radiolabelled albumin was displaced. When glycated human albumin was used as a competitor at the same concentration a similar proportion of the radiolabel was displaced. This indicates that native albumin and the glycated modified form of albumin have very similar affinities for this receptor. Neither lysozyme at 10x and 100x, nor immunoglobulin and α_1 acid glycoprotein at 10x displace significant amounts of [^{125}I]-albumin binding.

In contrast to the absent competitive effect of the various proteins, the polyanionic compounds heparin, polyglutamic acid, dextran sulphate and fucoidan each demonstrated a concentration dependent inhibition of [^{125}I]-albumin to OK cell monolayers. At 10x each of these compounds displaces approximately 25-30% of the binding of the radiolabelled

Figure 3.12
The Effects of Various Competitors on [^{125}I]-Albumin Binding to OK Cell Monolayers

The control condition represents binding in the presence of [^{125}I]-albumin alone. Non-specific binding values have been subtracted. The competitors are divided into two groups: protein competitors in the upper group of bars, and other anionic macromolecules in the lower group of bars. The cell monolayers were incubated with [^{125}I]-albumin at a concentration of 150 mg/L (2.2 μM). The other competitors were used at either a tenfold (10x) or 100-fold (100x) molar excess concentration compared to that of the radioligand. Values represent means ± SE; n ≥3 determinations performed in duplicate. * $p < 0.01$ compared to controls.


Control
Unlabelled Albumin(10x)
Glycated Albumin(10x)
Lysozyme (10x)
Lysozyme (100x)
Immunoglobulin (10x)
α1 Acid Glycoprotein (10x)
Heparin (10x)
Heparin (100x)
Polyglutamic Acid (10x)
Polyglutamic Acid (100x)
Dextran Sulphate (10x)
Dextran Sulphate (100x)
Fucoidan (10x)
Fucoidan (100x)
*
0
25
50
75
100
% Specific Albumin Binding

albumin, whereas at 100x these same compounds displace 60-70% of albumin binding.

3.3 Discussion

The binding experiments described in this thesis document two binding sites for albumin in OK cells as measured at 4°C. One site has a high affinity and low capacity, whilst the other has low affinity but high capacity. These results are qualitatively in agreement with those of other authors who examined albumin binding and uptake into isolated proximal tubular segments and OK cell monolayers (Park and Maack 1984, Schwegler *et al* 1991). These previous authors however performed their experiments at 37°C and hence the kinetic data they describe represent a combination of both binding and endocytosis of albumin. This observation would explain, at least in part, the differences in the K_D described for albumin binding in the current study and those previously described.. For instance Schwegler *et al* (1991) described a binding site in OK cells with a K_M of 24 mg/L as determined according to Michaelis Menton kinetics. These data refer to a transport process composed of binding to, and uptake of albumin into cells, rather than simply binding to cells as described in the present study. Schwegler *et al* (1991) were unable to identify a lower affinity site for albumin binding although the maximum incubated albumin concentration used in their study was 300 mg/L as opposed to 10 000 mg/L in our study.

Park and Maack (1984) have examined the kinetics of [^{3}H]-albumin binding to perfused proximal tubules isolated from rabbit kidneys. In this

model, albeit at 37°C, two potential albumin binding sites were resolved (K_M 31 mg/L and K_M 1.2g/L). In a similar manner to the results described above the values for K_M derived from these experiments represent a transport process rather than simply binding. Furthermore isolated proximal tubules possess a dense brush border which is lacking in OK cells, and therefore poorly defined steric factors make a direct comparison between these set of data difficult.

Gekle *et al* (1996) have examined binding affinities of albumin to OK cells at 4°C using relatively modest incubated albumin concentrations of up to 300 mg/L. These authors calculated the K_D for albumin binding to a single site as 20 mg/L after 15 minutes of binding. This value differs from the measured affinity of albumin for it's binding sites at 4°C documented in this thesis. However there is an important difference between the experimental conditions used in the experiments of Gekle *et al* (1996) and the experiments described in this thesis. The binding experiments in this thesis used an overnight incubation period, whereas Gekle *et al* (1996) used an incubation period of 15 minutes. The time course studies in this thesis show that albumin binding to OK cells at 4°C has not reached equilibrium at 15 minutes, and therefore this time point is inappropriate for the calculation of equilibrium binding constants.

The described affinities of albumin for these receptors are very much in keeping with the physiological albumin concentrations likely to be encountered by the apical membranes of proximal tubular cells *in-vivo*. A number of investigators have measured proximal tubular fluid albumin

concentrations in both normal and nephrotic rats (Baldamus *et al* 1975, Galaske *et al* 1978, Landwehr *et al* 1976, Lewy and Pesce, 1973, Oken and Flamenbaum 1971). The values measured have varied from 10 - 135 mg/L in normal rats, depending at least in part, on the assay used to measure the albumin . Such a concentration of albumin could be re-absorbed via the high affinity binding site documented in this study. In nephrotic animals however the proximal tubular fluid albumin concentration has been measured to be up to 150 times that seen in health. At albumin concentrations in this range the high affinity site would be fully saturated, but the low affinity high capacity albumin binding site documented in the present experiments would be available to effect the re-absorption of these larger quantities of albumin.

Ranganathan and Mego (1986) have also examined renal plasma membranes for the presence of receptors for modified serum albumins. Using predominantly basolateral derived membranes highly purified in Na^+, K^+-ATPase activity, these authors demonstrated high and low affinity binding sites for formaldehyde treated, reduced-carboxymethlyated, and reduced-acetamidated bovine serum albumins. The affinities of these binding sites were very similar to those described in the current experiments, but since our experiments examined binding of native albumin to apical rather than basolateral membranes of tubular epithelial cells the exact relationship of these two sets of binding data is unclear.

The nature of the albumin receptor in kidney proximal tubular cells has been obscure. The experiments described in the current study suggest

that the receptor(s) is a glycoprotein. Binding of albumin to OK cells can be inhibited by the lectins GM and UE by more than 50%. This inhibition is totally reversed by pre-incubation of the lectin with it's cognate hapten. These results indicate that the albumin receptor is glycosylated and possesses O-linked, but not N-linked glycans. More importantly however for the purposes of the current experiments, these results indicated that these inhibitory lectins may be useful to further investigate the properties of the albumin receptors.

In a further attempt to characterise the albumin receptor in OK cells the technique of ligand blotting has been employed. This procedure depends on the preservation of specific protein/protein interactions after electrophoresis and blotting, and is particularly useful for the identification of receptors when no anti-receptor antibodies are available (Daniel *et al* 1983). Using this technique a number of specific albumin binding proteins in OK cells have been identified. The ability of inhibitory lectins which block [^{125}I]-albumin binding to OK cell monolayers also to block binding of [^{125}I]-albumin to ligand blots suggests strongly that the proteins observed to bind albumin in the ligand blots are the same as those binding [^{125}I]-albumin to OK cell monolayers. This view is strengthened by the observation that the lectins which failed to inhibit [^{125}i]-albumin binding to cell monolayers also failed to inhibit it's binding to ligand blots. Furthermore only the inhibitory lectins bound to similar protein bands to [^{125}I]-albumin in the ligand blots. Based on these observations it seems highly likely that the proteins detected on nitrocellulose membranes by the

peroxidase labelled lectins are identical to those which bind albumin in the ligand blots, and that these in turn are the same proteins which bind [^{125}I]-albumin to OK cell monolayers in culture.

Several investigators have examined microvascular endothelial cells by ligand blotting in order to identify possible albumin receptors (Ghinea *et al* 1988, Schnitzer *et al* 1988, Ghinea *et al* 1989, Schnitzer *et al* 1990a, Schnitzer *et al* 1990b, Schnitzer 1992, Schnitzer *et al* 1992, Schnitzer and Bravo 1993, Schnitzer and Oh 1994, Tirrupathi *et al* 1995). In these cells potential glycoprotein (gp) receptors for various albumins demonstrated have been designated gp14, gp18, gp30, gp31 and gp60, where the number denotes the molecular weight in kilodaltons. The gp18 protein is also present on a variety of tumour cells (Wang *et al* 1994). Of these proteins the gp60 protein has been particularly well characterised. It carries O-linked but not N-linked glycans, is antigenically related to glycophorin, and has been christened "albondin" (Schnitzer *et* al 1990a, Schnitzer *et* al 1994, Tiruppathi *et al* 1995). This protein appears to function as a specific receptor for native unmodified albumin, which has been postulated to have an important role in the regulation of vascular permeability, and has been detected particularly in tissues possessing a continuous endothelium. The experiments described in this study have demonstrated that albumin labels only very weakly a band at 60 kDa in the ligand blots of OK cell membranes, and therefore albondin does not appear to be the major mediator of albumin binding to these cells.

The gp14, gp18 and gp30 albumin binding proteins detected in the OK cell membranes on the other hand demonstrate both marked differences and similarities to those proteins of identical molecular weight detected by Schnitzer et al (1992), and Schnitzer and Bravo (1993), which mediate binding of albumin to endothelial cells. These proteins were originally described in endothelial cells (Ghinea *et al* 1988, Schnitzer *et al* 1988, Ghinea *et al* 1989, Schnitzer 1992) although it subsequently became clear that the same proteins were present in other cells associated with the vascular wall, notably smooth muscle and fibroblast cells. Epithelial cells have not been previously studied in isolation, although a crude tissue extract derived from whole rat kidney was found to contain albumin binding proteins of 18 and 30 kDa when ligand blotted with bovine serum albumin (BSA) modified by colloidal gold conjugation. Kidney tissue comprises a heterogeneous population of cell types, and therefore such experiments give no indication of the precise site of expression of a receptor. Very recently support for this data has come from the work of Cessac-Guillemet *et al* (1996). Using affinity chromatography of rat renal brush border membranes on rat serum albumin-Sepharose columns, this group of workers were able to identify both a 31 kDa and a 55 kDa albumin binding protein in this nephron segment. Antibodies raised to these proteins demonstrated the presence of these proteins all along the proximal tubule.

Other investigators have suggested that the major physiological roles of gp18 and gp30 in endothelium are as scavenger receptors mediating of binding and subsequent endocytosis of modified albumins

followed by their degradation. Similar proteins may also be present in liver cell membranes to facilitate the absorption of chemically modified albumin (Ottnad *et al* 1990). In support of this hypothesis is the observation that in ligand blotting experiments the chemically modified albumins, maleic anhydride-BSA and formaldehyde-BSA, bound to gp18 with a K_D of 5 and 90 μg/ml respectively, and to gp30 with a K_D of 4 and 75 μg/ml respectively. In contrast, chemically unmodified native BSA demonstrated much lower affinities, with a K_D 4 and 5 mg/ml for gp30 and gp18 respectively (Schnitzer *et al* 1992).

The K_D for [^{125}I]-albumin binding to the high affinity site in OK cell monolayers in our experiments was approximately 150 mg/L. The binding affinity of [^{125}I]-albumin to the ligand blots was very similar and it seems likely therefore that the albumin binding proteins detected by ligand blotting represent the higher affinity site detected in OK cell monolayers. In addition it has been previously demonstrated radio-iodination of albumins does not alter their ligand binding behaviour (Schnitzer *et al* 1990a). Clearly therefore the affinity of the gp18 and gp30 for unmodified albumin in the OK cell membranes is substantially higher than in other systems previously examined.

The lectin affinities of gp18 and gp30 described by Schnitzer *et al* (1992) are somewhat different than the lectin affinities of the gp18 and gp30 demonstrated in our experiments. For instance in Schnitzer's experiments only Con A was able to inhibit colloidal gold-albumin binding to ligand blots of endothelial cell proteins, and this same lectin, along with

a number of others, was able to bind to blotted endothelial cell proteins (Schnitzer *et al* 1992). In our experiments however Con A was unable to inhibit albumin binding to OK cell monolayers or ligand blots, and was similarly incapable of binding to gp18 and gp30 in lectin blots of OK cell membrane proteins. These results indicate that if the gp18 and gp30 found in endothelial cells are the same as those in OK cells then they must be differently glycosylated.

The effects of the various competitors on albumin binding to the OK cell monolayers agrees closely with similar competition studies which examined the potential competitors of modified albumin binding to gp18 and gp30 in ligand blotting experiments. The binding of albumin to OK cell monolayers and endothelial cell gp18 and gp30 was competed similarly by a number of polyanionic macromolecules. These molecules have previously been demonstrated to interact with some types of scavenger receptors (Schnitzer and Bravo 1993, Krieger and Herz 1994, Westwood *et al* 1994).

Various protein competitors show no affinity for the albumin binding sites in OK cell monolayers. The competitor proteins employed in these experiments are all components of normal or inflamed urine (Bergarrd 1970) and may logically have been predicted to compete for any non-specific protein binding sites in proximal tubular cells. The results of the current studies therefore suggest that the albumin receptor is highly specific for this protein. Furthermore this pattern of protein ligand specificity is also very much in keeping with the previously described

affinities of some scavenger receptors (Schnitzer and Bravo 1993, Kreiger and Herz 1994).

In contrast to the current findings the endothelial cell gp18 and gp30 bind native or glycated albumins much less avidly than chemically modified albumins. The binding of chemically modified albumins to OK cells has not been examined in this thesis, but it is possible that these molecules may bind with even higher affinity than does native albumin. In the competition experiments documented in this thesis both unlabelled native albumin and unlabelled glycated albumin compete the binding of [^{125}I]-albumin to a similar degree. This suggests that both native and glycated albumin have similar affinities for the binding site in OK cells. It is of interest therefore to note the results of Bendayan and Londono (1996) which documented preferential re-absorption of native albumin with respect to glycated albumin in the normal mouse proximal tubule. These authors assessed albumin uptake in a morphological study of mice injected with hapten tagged albumins. Binding of albumins to proximal tubular cells was not measured.

Nonetheless the notion of a scavenger receptor mediating albumin binding and uptake in the renal proximal tubule makes much sense. Classically scavenger receptors are thought to mediate the uptake of modified proteins that are destined to undergo lysosomal degradation in the cell (Schnitzer and Bravo 1993, Kreiger and Herz 1994). This is precisely the fate of the majority of approximately 9g of native albumin (assuming a glomerular filtration rate of 125 ml/min and glomerular filtrate

albumin concentration of 50 mg/L) filtered into the mammalian proximal tubule each day. This albumin, although thought to be unmodified, is not recycled in intact form to the circulation. Rather, it is re-absorbed into proximal tubule epithelial cells by endocytosis and broken down completely to it's constituent amino acids which are then re-absorbed into the blood (Park and Maack 1984, Carone *et al* 1979).

Therefore in summary the presence on renal epithelial cell surfaces of albumin binding sites which have ligand specificities very similar to some types of scavenger receptors, and molecular weights identical to albumin binding glycoproteins present in endothelial cells, has been documented. These results suggest that albumin is re-absorbed from the kidney proximal tubule by a scavenger receptor(s), and that this receptor may be the same protein that is found in other cells types in other tissues.

Chapter 4

Results 2

THE PROTEIN ENDOCYTIC FUNCTIONS OF OK CELLS

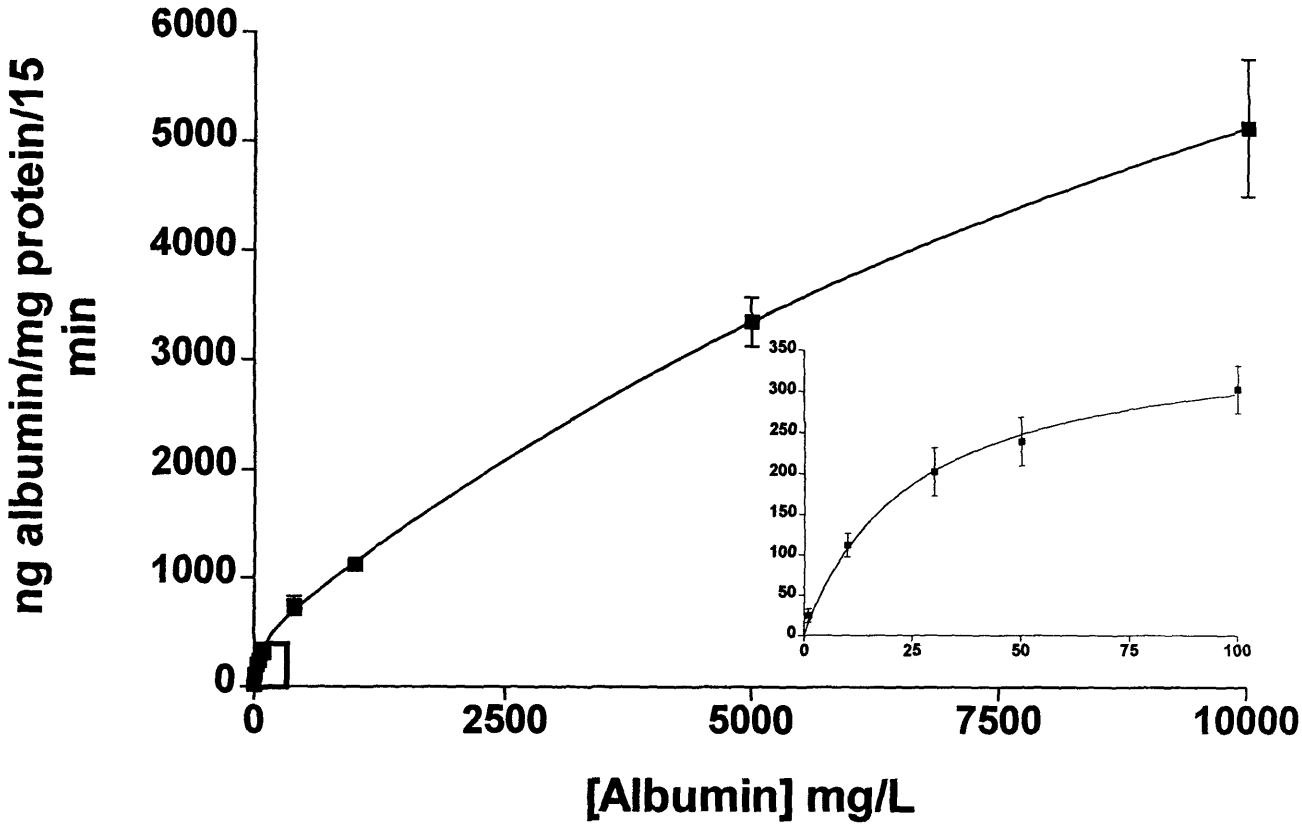
In contrast to the experiments detailed in the previous Chapter the following experiments were all performed at 37°C. At this temperature [^{125}I]-albumin does not simply bind to the cells but is also endocytosed by the cells.

4.1 Receptor Mediated Endocytosis of [^{125}I]-Albumin by OK Cells

In qualitative terms the process of [^{125}I]-albumin uptake into wild type OK cells at 37°C demonstrates saturation kinetics consistent with a receptor mediated process. The concentration dependence of [^{125}I]-albumin uptake into wild type OK cells at 37°C is shown in Figure 4.1. Two distinct phases of uptake are apparent. An early phase is seen which appears to saturate at low concentrations of incubated [^{125}I]-albumin. This is followed by a second phase which begins to saturate at much higher concentrations of incubated [^{125}I]-albumin. Analysis of these data by non-linear curve fitting confirms the presence of two potential uptake systems for [^{125}I]-albumin, one with high affinity for it's albumin substrate, but low

Figure 4.1
Concentration Dependency of [^{125}I]-Albumin Uptake into OK Cells.

Wild type OK cells were grown to confluence in 6 well plates. Cells were incubated with various concentrations of [^{125}I]-albumin at 37°C for 15 minutes. Values represent means ± SE; n ≥ 4 experiments performed in duplicate. Inset: uptake at lower concentrations of incubated [^{125}I]-albumin corresponding to boxed area in main graph. Non-specific binding of [^{125}I]-albumin was measured in the presence of a large excess of unlabelled albumin, and these values have been subtracted from this data.


ng albumin/mg protein/15 min
6000
5000
4000
3000
2000
1000
0
0
2500
5000
7500
10000
[Albumin] mg/L
350
300
250
200
150
100
50
0
0
25
50
75
100

capacity, and the other with lower affinity for substrate but much higher capacity. The apparent K_M of the high affinity uptake system is 24.3 mg/L (360 nM). This value approximates closely to the proximal tubular albumin concentration as previously measured by other investigators. The apparent K_M of the low affinity site is 15.9 g/L (88.6 μM).

However these calculated values for K_M can only be regarded as apparent estimates since under the conditions of these experiments the measured [^{125}I]-albumin uptake represents a composite of both binding of [^{125}I]-albumin to the cell surface, and subsequent endocytosis by receptor mediated and fluid phase systems. Despite this caveat however, the figures derived from the uptake data are broadly in agreement with other published values (Park and Maack 1984, Schwegler *et al* 1991).

4.2 Visualisation of Gold-Albumin Endocytosis by Electron Microscopy

In order to confirm the endocytic uptake of albumin in OK cells, the uptake of gold-albumin by confluent cells grown on coverslips was visualised by electron microscopy. Representative sections are depicted in Figure 4.2. After 15 minutes exposure to gold-albumin in serum free DMEM-F12 at 37°C a number of gold particles are seen bound to the plasma membrane. These bound particles appear to concentrate in plasma membrane invaginations and scanty gold particles can be observed in early endosomal structures in close association with the plasma membrane (Figure 4.2.A). In many instances the apical invaginations in which the gold-albumin is seen to be bound appear to be coated, and most likely

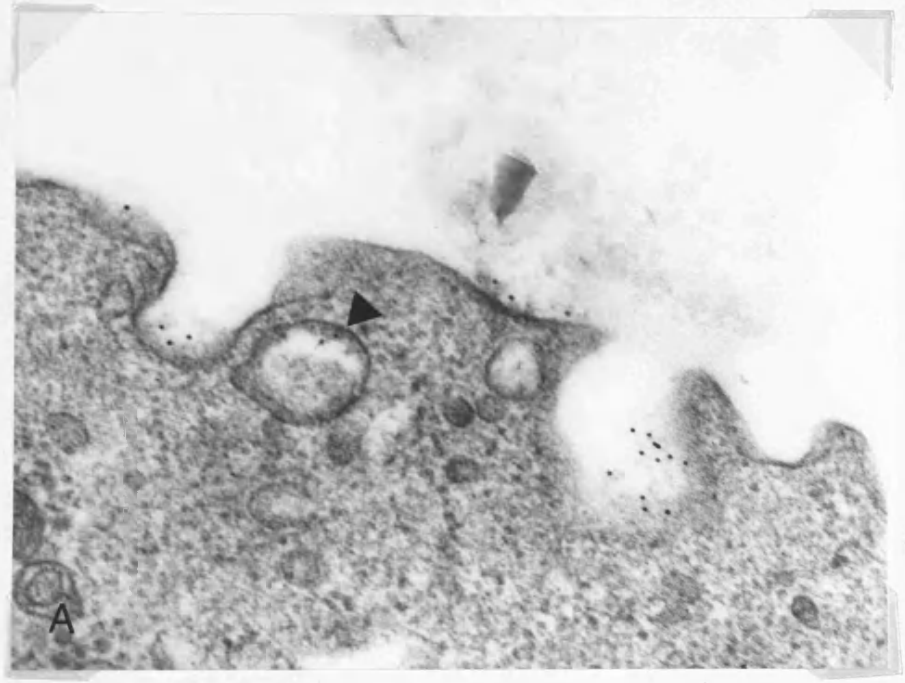
represent clathrin coated pits. This is particularly well demonstrated in Figure 4.2.B where a number of gold-albumin particles are seen to be present within a well developed coated pit on the apical cell surface. Numerous gold-albumin particles are also seen in an early endosomal structure within the same cell. In Figure 4.2.C gold-albumin particles are again demonstrated in an apical coated pit but are also present within a multivesicular body, a component of the later endosomal pathway. Multivesicular bodies are a particularly prominent feature of the apical vesicular apparatus in these OK cells. A more electron dense vesicular structure is also seen to contain a concentration of gold-albumin particles. This structure most likely represents a lysosome. Figure 4.2.D depicts the extensive apical tubulo-vesicular system present in OK cells together with the presence of gold-albumin particles in a multivesicular body. This phenomenon is again demonstrated in Figure 4.2.E where multiple apical vesicular structures are observed in a single OK cell with gold-albumin particles present in a multivesicular body and an electron dense vesicle, probably a lysosome. As mentioned above the multivesicular body is prominent in these OK cells, and a high power view of one of these structures is depicted in Figure 4.2.F. This structure contains many gold-albumin particles together with multiple fragments of invaginated membrane. It is interesting to note that at this time point no gold particles appear free in the cytoplasm, and all intracellular gold is within vesicular structures.

Figure 4.2
Electron Micrographs of Gold-Albumin Endocytosis by Wild Type OK Cells

OK cells were grown to confluence on coverslips and incubated with 10 nm gold-albumin for 15 minutes at 37°C. Cells were then fixed and processed for electron microscopy. Gold-albumin particles are represented as black spots.

A. Numerous gold-albumin particles congregating in OK cell apical invaginations. Several gold-albumin particles are present in an endosomal vesicle (arrowhead). x 63 500.

B. Gold-albumin particles are seen to be bound in a well developed OK cell apical invagination which appears to be coated, most likely with clathrin. Further gold-albumin particles are present in an intracellular endocytic vesicle. N = nucleus. x 67 500.


A

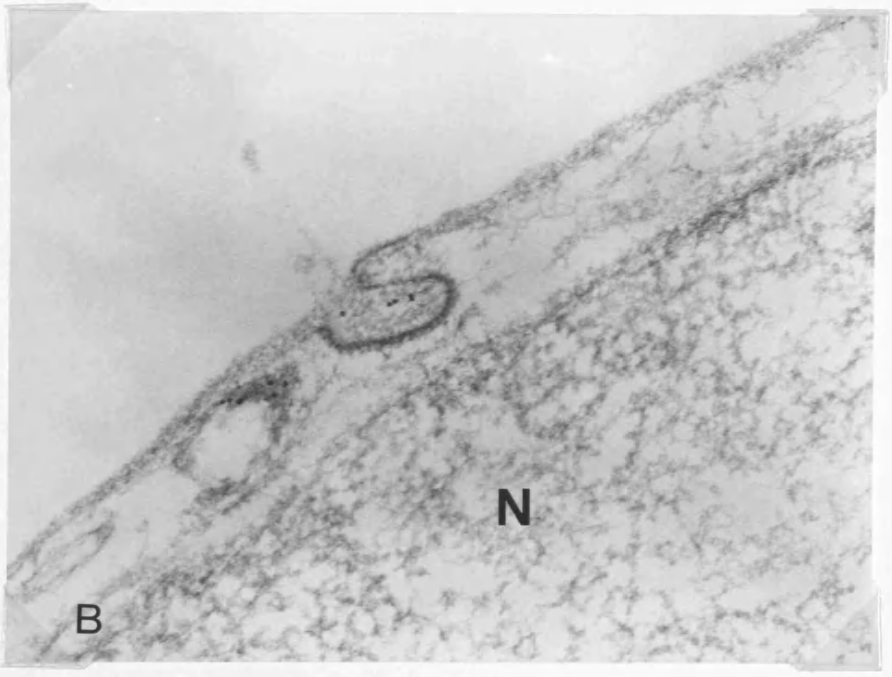
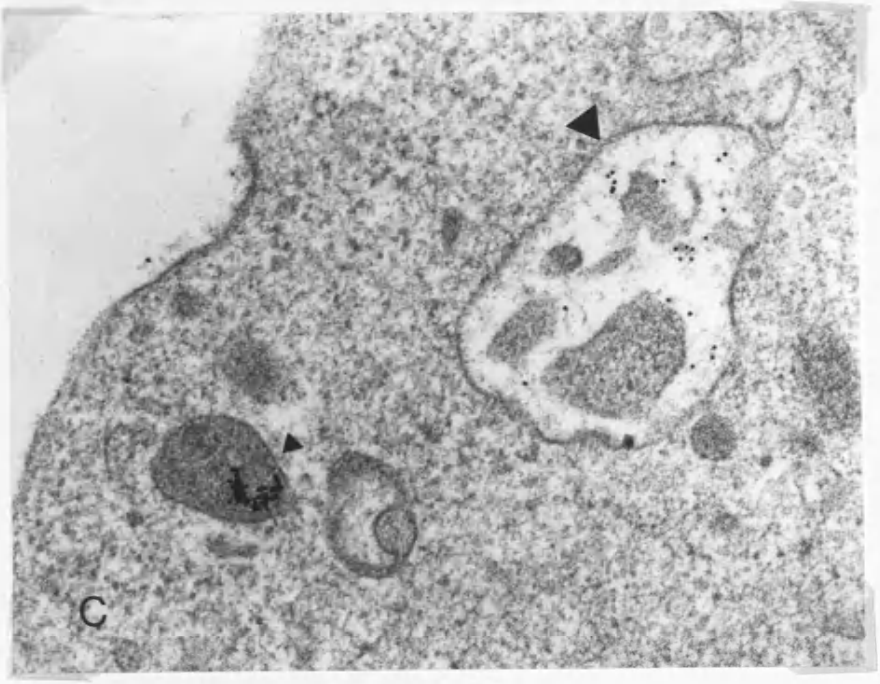

N
B

Figure 4.2 continued
Electron Micrographs of Gold-Albumin Endocytosis by Wild Type OK Cells

C. Gold-albumin particles are seen to be present in all components of the endocytic pathway. Two particles are present in a coated apical invagination. There are a number of gold-albumin particles to be seen in a multivesicular body which contains multiple pieces of plasma membrane endocytosed from the apical surface of the cell (large arrowhead). Many gold-albumin particles are seen concentrated in an electron dense structure which probably represents a lysosome (small arrowhead). x 64 000.

D. Two adjacent OK cells with the tight junction indicated (arrowhead). The cell on the left contains a multivesicular body which in turn contains around 20 gold-albumin particles. These cells also possess a number of other vesicular structures none of which contain gold-albumin. x 55 000.


C

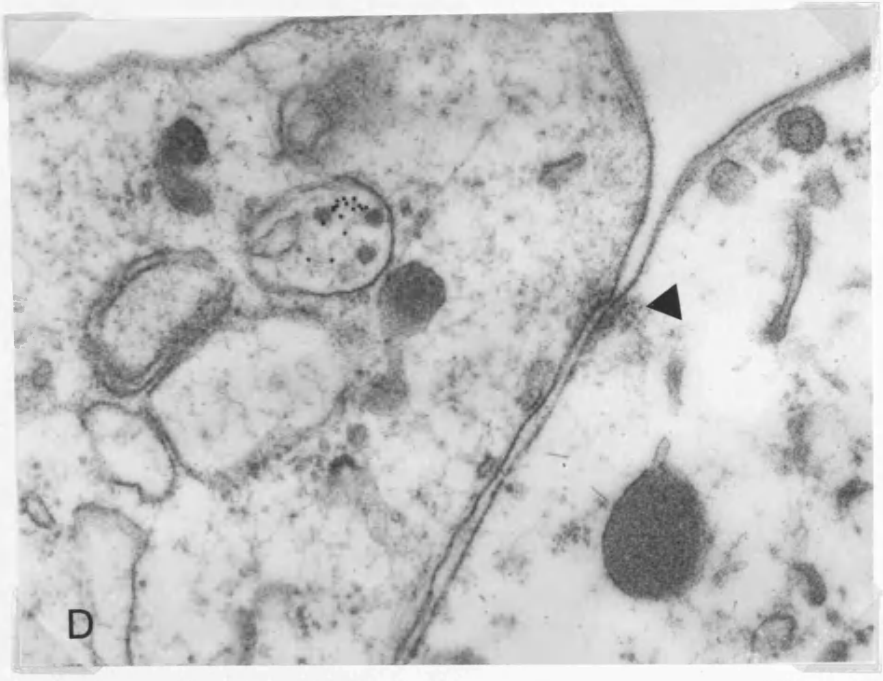
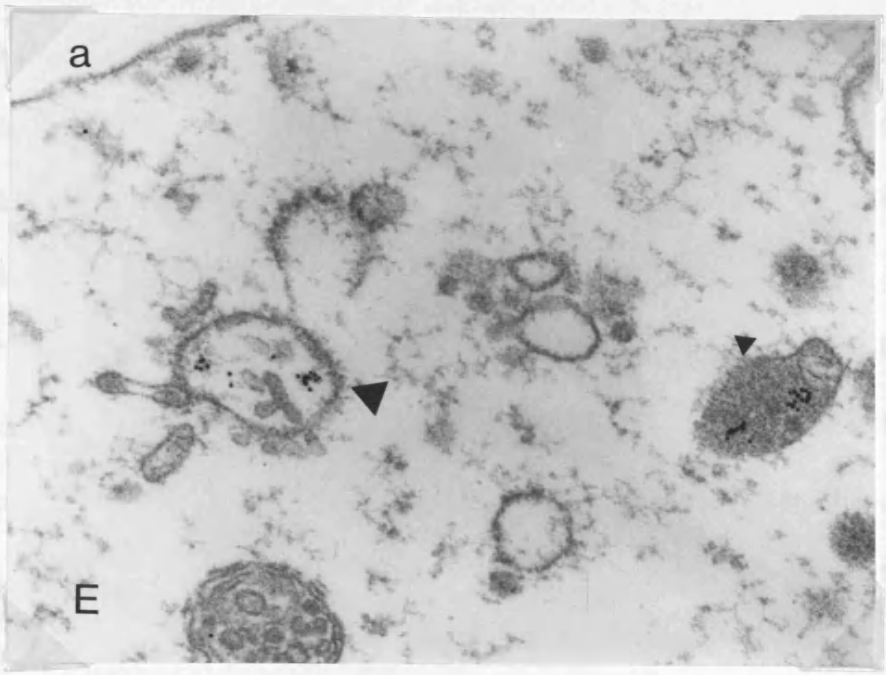
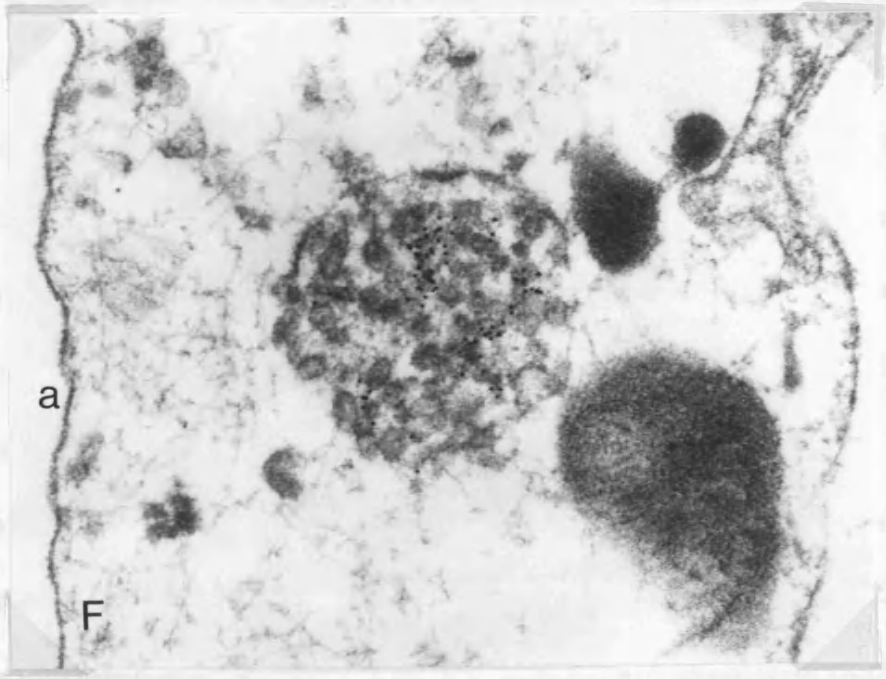

D

Figure 4.2 continued
Electron Micrographs of Gold-Albumin Endocytosis by Wild Type OK Cells

E. OK cell cytoplasm demonstrating the presence of numerous vacuolar structures. Gold-albumin particles are present in a multivesicular body (large arrowhead) and a late endosomal structure, most likely a lysosome (small arrowhead). a = apical membrane, x 60 000.

F. Large number of gold-albumin particles present in an OK cell multivesicular body. a = apical membrane, x 59 000.


a
E


a
F

In control experiments cells were incubated with gold-albumin in the presence of a 1000 fold excess of unlabelled albumin. Under these experimental conditions only very scanty gold particles were observed bound to the cell surface, representing non-specific binding. In control experiments gold particles were not visualised in any intracellular vesicular organelles.

The electron microscopic experiments therefore demonstrate an extensive apical vesicular compartment in OK cells. Albumin is endocytosed into this vesicular compartment through coated pits, and following it's endocytosis albumin is seen in early, intermediate, and late components of the endosomal-lysosomal pathway.

4.3 NEM Sensitive Uptake of [^{125}I]-Albumin by OK Cells

Pre-incubation of cells with NEM had a marked effect on [^{125}I]-albumin endocytosis. This agent has been used to inactivate $G\alpha_i$ mediated responses in many systems (Jakobs *et al* 1982, Smith and Harden 1984, Wong *et al* 1985), but can also interact with other components of the endocytic pathway. When OK cells were incubated with increasing concentrations of NEM, ranging from 1μM to 1mM, a dose dependent reduction in the endocytosis of 50 mg/L [^{125}I]-albumin was observed. The IC_{50} for this effect of NEM was approximately 20.0 μM (Figure 4.3).

OK cells were then pre-treated with 100 μM NEM and allowed to endocytose various concentrations of [^{125}I]-albumin for 15 minutes. Under these conditions a marked and significant reduction in albumin endocytosis

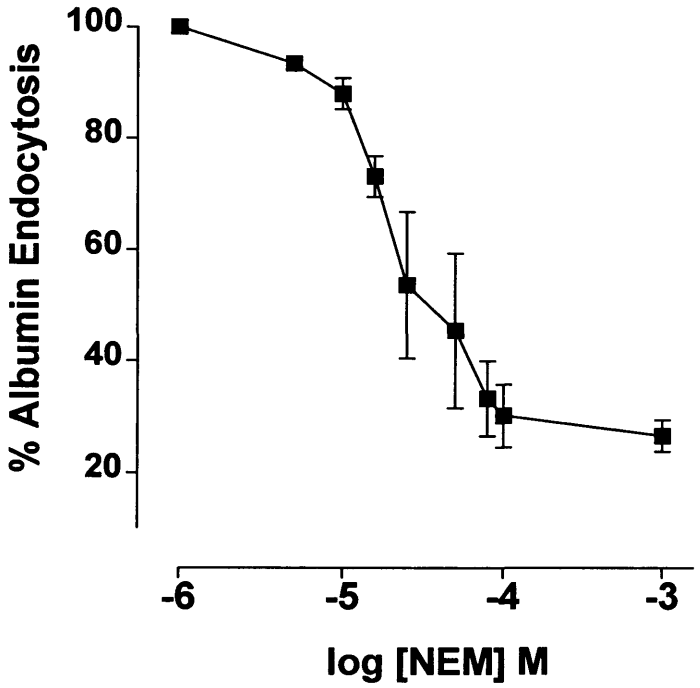


Figure 4.3
Dose Response Effect of N-Ethylmaleimide on Endocytosis of [^{125}I]-Albumin by Wild Type OK Cells

Confluent wild type OK cells were grown in 6 well plates. Cells were exposed to various concentrations of NEM prior to the initiation of [^{125}I]-albumin endocytosis for 15 minutes at 37°C. Cell monolayers were washed and the extract counted in a gamma counter. Values representing binding have been subtracted. Values of 100% represent endocytosis by control, non-NEM treated OK cells. Values are mean ± SE; n = ≥3 experiments performed in duplicate.

was observed at each incubated albumin concentration, except the lowest 1 mg/L concentration, compared to controls (Figure 4.4).

4.4 Regulation of Endocytosis by Heterotrimeric GTP-Binding Proteins

In order to further investigate the regulation of the endocytic pathway, cell monolayers were incubated with G-protein modulating agents prior to the initiation of [^{125}I]-albumin binding and uptake. These results are depicted in Figure 4.4. A striking effect is seen when OK cell monolayers are pre-incubated with pertussis toxin overnight. This bacterial toxin ADP-ribosylates and inactivates the alpha subunits of the $G\alpha_i$ and $G\alpha_o$ (Cote *et al* 1984) families of heterotrimeric G-proteins, whilst having no effect on the function of the members of other families. OK cell [^{125}I]-albumin uptake is considerably reduced by more than 50% under these conditions, this effect being seen at all concentrations of incubated albumin examined (Figure 4.4).

This phenomenon was not a result of reduced binding of [^{125}I]-albumin to the cells in the presence of the bacterial toxin. Pertussis toxin pre-incubation did not alter or inhibit either the specific or non-specific binding of [^{125}I]-albumin to OK cells as measured at 4°C (Figure 4.5).

To further characterise potential G-protein regulation of the endocytic pathway in these cells some monolayers were pre-incubated overnight with cholera toxin. This bacterial toxin ADP-ribosylates the alpha subunits of the G_S family of G-proteins rendering them constitutively active (Gill and Merem 1978). However this experimental manoeuvre had no

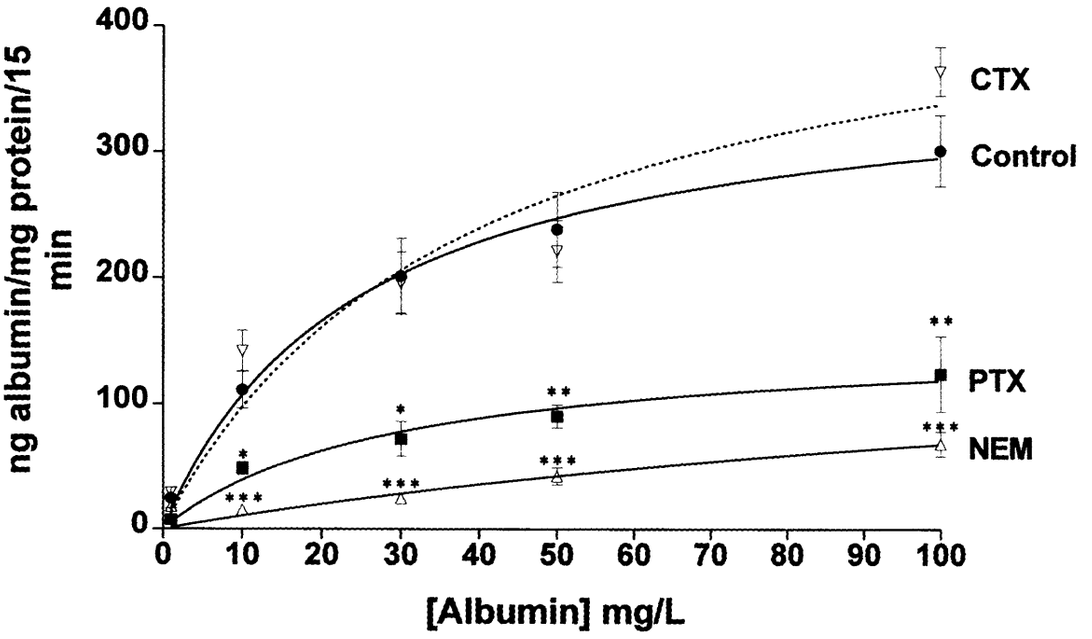


Figure 4.4
Effects of Vesicular Transport and G-Protein Modulating Agents on Albumin Endocytosis by Wild Type OK Cells

[^{125}I]-albumin uptake was measured in wild type OK cells after 15 minutes incubation at 37°C, under control conditions (●) or after pre-treatment with NEM (Δ), pertussis toxin (■) or cholera toxin (∇, dashed line). Values for non-specific binding of albumin have been subtracted. Values are means ± SE of at least 3 experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$, and *** < 0.001 compared to controls.

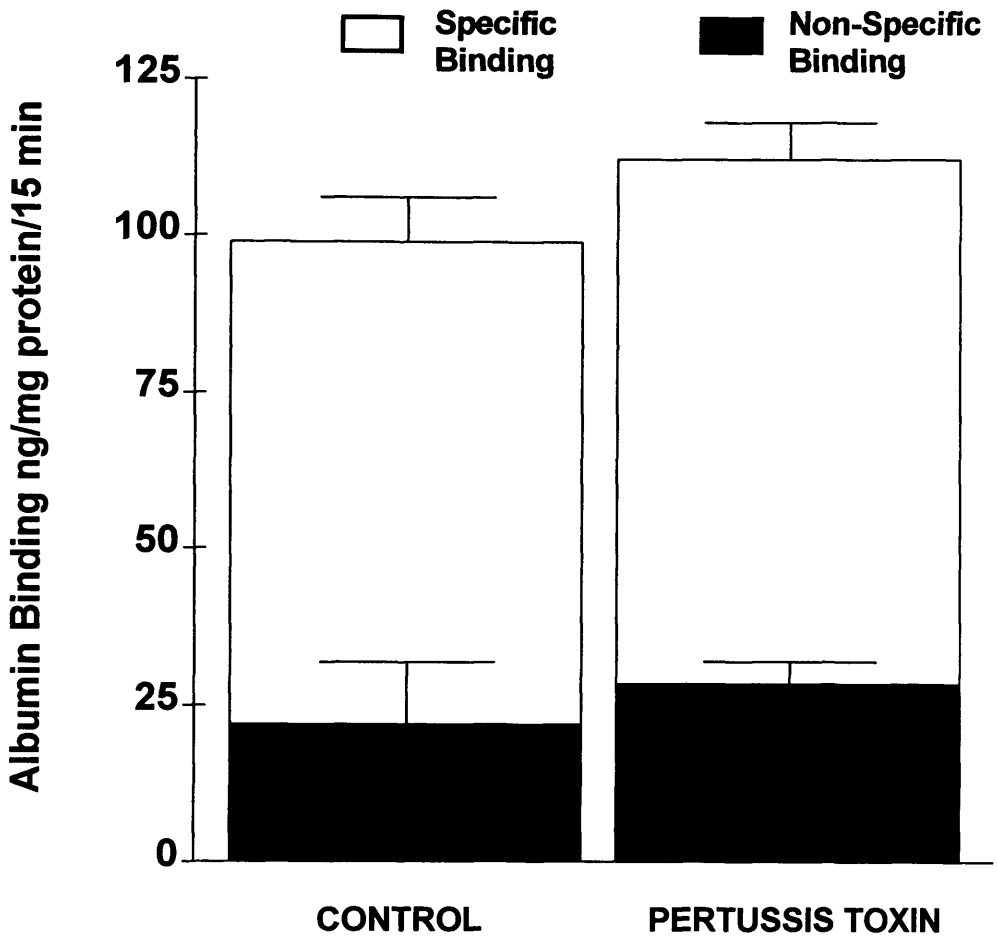


Figure 4.5
[^{125}I]-Albumin Binding to Control and Pertussis Toxin Treated OK Cells

OK cells were grown to confluence in 6 well plates and incubated with 50 mg/L [^{125}I]-albumin for 15 minutes at 4°C. Non-specific binding was defined in the presence of a large excess of unlabelled human albumin. Binding experiments were performed using untreated control wild type OK cells, or wild type OK cells pre-treated overnight with 100 ng/ml pertussis toxin. Values are means ± SE; n = 3.

significant effect on [^{125}I]-albumin uptake in these cells (Figure 4.4). There was a tendency towards higher levels of uptake at higher concentrations of incubated albumin but this failed to reach statistical significance.

4.5 *Fluid Phase Endocytosis by OK Cells*

Extracellular fluid is trapped inside coated and non-coated vesicles when they invaginate to form vesicles. Substances dissolved in the extracellular fluid therefore undergo obligate internalisation. This process is known as cell drinking or fluid phase endocytosis. To determine the fraction of total endocytosis that was contributed by the fluid phase OK cells were incubated with [^{3}H]-inulin dissolved in serum free DMEM F12. Inulin is a suitable marker to measure this mode of endocytosis since the proximal tubular cells possess no receptors for it, and inulin does not therefore bind to the cells. Thus any absorption can only occur via fluid phase endocytosis. In contrast to the uptake of [^{125}I]-albumin, [^{3}H]-inulin uptake is not a saturable process. The uptake of [^{3}H]-inulin increases in a linear manner with increasing concentration in the incubating solution as would be expected of a fluid phase marker. Furthermore [^{3}H]-inulin showed considerably less uptake. For instance after 15 minutes at 37°C 50 mg/L [^{125}I]-albumin displays approximately 10 fold greater uptake than does 50 mg/L [^{3}H]-inulin

Pre-treatment of cells with PTX also resulted in reduced [^{3}H]-inulin uptake. However due to the much smaller total quantities involved this phenomenon was only statistically significant at the 30 mg/L incubated [^{3}H]-inulin concentration (Figure 4.6).

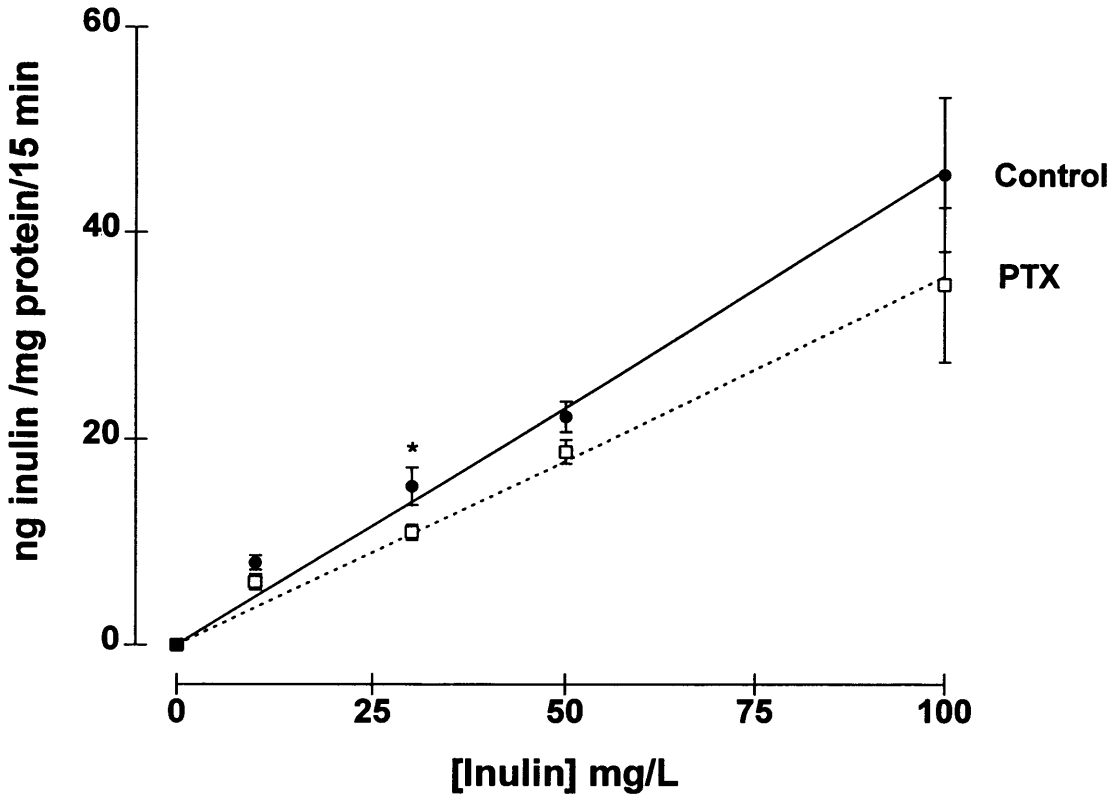


Figure 4.6
Fluid Phase Endocytosis of Inulin in OK Cells

Wild type OK cells were grown to confluence in 6 well plates. Fluid phase endocytosis was measured using [^{3}H]-inulin as a marker. Various concentrations of [^{3}H]-inulin were incubated with the cells for 15 minutes at 37°C. The cells used were either wild type untreated control OK cells (solid line), or wild type OK cells pre-treated with 100 ng/ml pertussis toxin (dashed line) overnight. Values are means ± SE of at least 3 experiments performed in duplicate. * < 0.05 compared to controls.

4.6 Summary

The results of this set of experiments therefore suggested that albumin bound to two distinct sites on OK cells and was subsequently taken up into the cell by receptor mediated endocytosis. Experiments with pertussis and cholera toxins implied a role for G-proteins in the regulation of the endocytic mechanism. More specifically inhibition of albumin uptake by pertussis toxin favoured a regulatory role for the $G\alpha_i$ or $G\alpha_o$ family of G-proteins. The lack of response to cholera toxin suggested that the $G\alpha_S$ family of G-proteins were not directly regulating the endocytic pathway.

Transfection of OK cells with the cDNA for $G\alpha_{i-3}$ was therefore performed in order to evaluate the hypothesis that the regulation of the endocytic pathway was by the $G\alpha_{i-3}$ protein.

4.7 Overexpression of $G\alpha_{i-3}$ Protein in OK Cells

4.7.1 *Sub-cloning of DNA and preparation of eukaryotic expression vectors*

E coli strain BL21-DE3 expressing the plasmid NpT7-5/$G\alpha_{i-3}$ were grown in 200 ml of ampicillin containing NZY culture broth overnight and the plasmids prepared in a large scale preparation as described in **2.14.5**. The plasmid DNA obtained from this preparation was re-suspended in 500 µl of sterile H_2O. A 3µl aliquot of this preparation was then subjected to digestion with the restriction enzymes *Hind III* and *EcoRI* to excise the $G\alpha_{i-3}$ cDNA insert. The resulting DNA fragments were then separated by agarose gel electrophoresis. Two fragments were obtained, a 1.7 kb

fragment representing the $G\alpha_{i-3}$ cDNA insert, and a heavier fragment representing the digested NpT7-5 plasmid (Figure 4.7). Therefore a further digest of 13 µl of the plasmid DNA was performed and the 1.7 kb fragment separated form the heavier fragment by agarose gel electrophoresis and purified as described in **2.14.8**.

Then 15 µg of the plasmid pBSII SK were digested with *HindIII* and *EcoRI*. The resulting linearised plasmid was then gel purified. A ligation reaction containing 30 ng of *HindIII/EcoRI* digested plasmid pBSII SK, and 51 ng of *HindIII/EcoRI* $G\alpha_{i-3}$ cDNA fragment was then performed. The products of this reaction were then transfected into competent E coli strain DH5α. Successful transfection of these E coli with re-circularised plasmid pBSII SK/ $G\alpha_{i-3}$ was expected to render the bacteria ampicillin resistant. Therefore the transfected bacteria were plated onto ampicillin containing culture plates and incubated overnight. As a control some bacteria were transfected with linearised plasmid pBSII SK which had been subjected to the ligation reaction conditions but in the absence of the $G\alpha_{i-3}$ cDNA fragment. Multiple bacterial colonies grew on the pBSII SK/$G\alpha_{i-3}$ plate, but none were present on the control plate.

Ten colonies were picked off the pBSII SK/$G\alpha_{i-3}$ plate, expanded and used for plasmid mini-preparations as described in **2.14.6**. A 10µl aliquot of the plasmid preparation was then digested with *HindIIi* and *EcoRI* to ensure that the plasmids derived from the preparation contained the $G\alpha_{i-3}$ cDNA insert. As can be seen in Figure 4.8 the plasmids derived from the 10 individual plasmid mini-preparations each carried the $G\alpha_{i-3}$ cDNA insert.

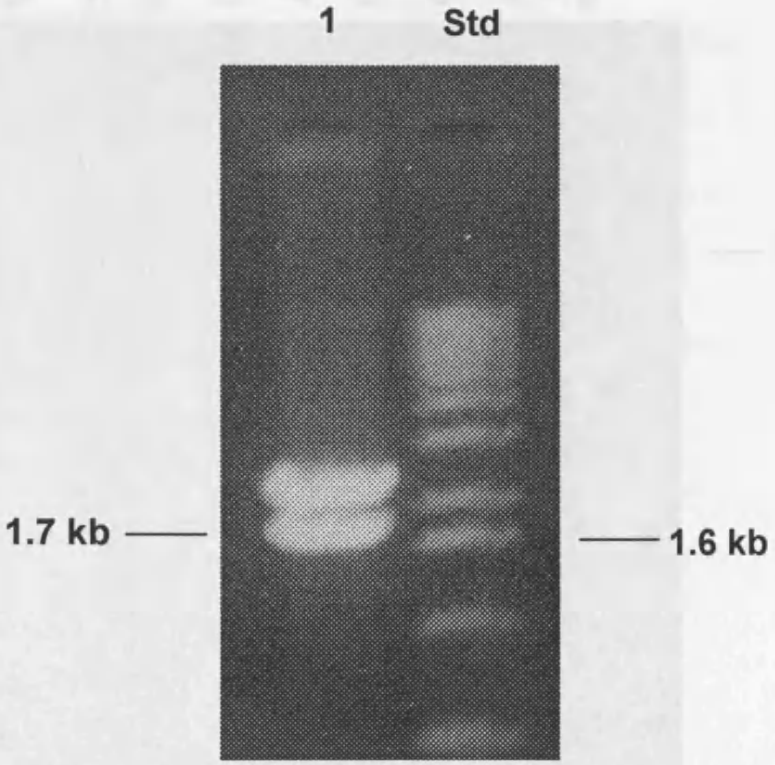


Figure 4.7
Ethidium Bromide Agarose Gel Electrophoresis of NpT7-5/Gα_{i-3} *Hind III/EcoR1* Digest

3 μl of plasmid NpT7-5/Gα_{i-3} was digested with *Hind III* and *EcoRI* , and the resulting plasmid DNA fragments separated by ethidium bromide agarose gel electrophoresis. The gel was exposed to UV light and photographed. Lane 1 represents digested plasmid DNA; Std lane represents DNA ladder standards with a 1.6 kb DNA standard band indicated. The 1.7 kb fragment in lane 1 is the excised Gα_{i-3} cDNA insert. The heavier band in lane 1 is the linearised NpT7-5 plasmid from which the Gα_{i-3} cDNA has been excised.

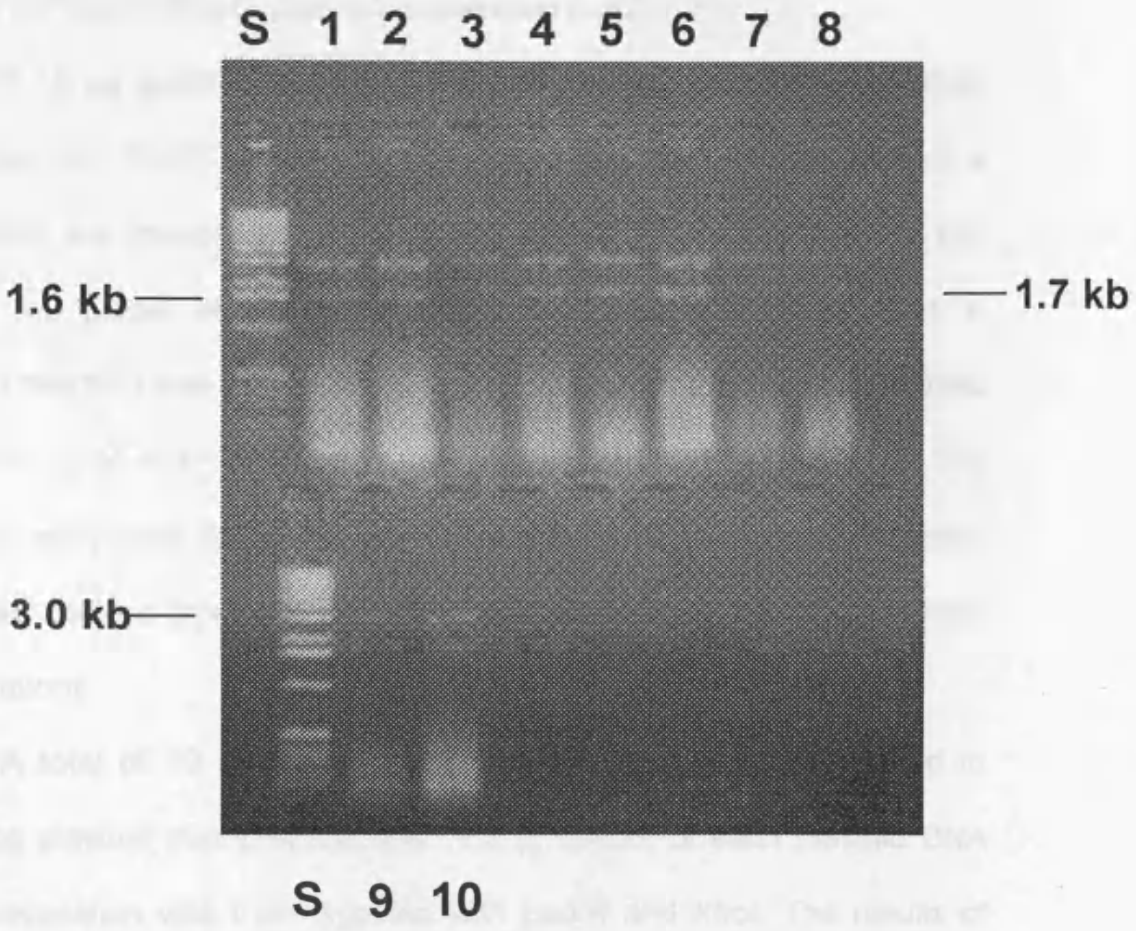


Figure 4.8
Ethidium Bromide Agarose Gel Electrophoresis of pBSII SK/Gα_{i-3} Plasmid Mini-Preps Digested with *HindIII*/*EcoRI*

Plasmid mini-preps were prepared from 10 individual penicillin resistant colonies of DH5α previously transformed with plasmid vector construct pBSII SK/Gα_{i-3}. A 10 μl aliquot of each of these preparations was then digested with the restriction endonucleases *HindIII* and *EcoRI*. The resulting digested DNA fragments were separated by ethidium bromide agarose gel electrophoresis, exposed to UV light and photographed. Lane S = DNA ladder standards with the 3.0 and 1.6 kb standard bands indicated. Digestion of plasmids derived from each of the 10 bacterial colonies yields a 1.7 kb fragment representing Gα_{i-3} cDNA fragment, and a heavier ~3.0 kb fragment representing digested linearised pBSII SK.

A large scale plasmid preparation was then performed using the reference culture corresponding to plasmid preparation number 2.

A 10 μg quantity of both the pBSIISK/$G\alpha_{i-3}$ and pcDNA3 were then digested with *EcoRI* and *XhoI*. This yielded linearised pcDNA3 and a restriction site compatible $G\alpha_{i-3}$ cDNA fragment (Figures 4.9 and 4.10). These two pieces of DNA were then gel purified and electroeluted. A ligation reaction was set up using 30 ng of *EcoRI/XhoI* digested pcDNA3 and 70 ng of *EcoRI/XhoI* digested $G\alpha_{i-3}$ cDNA. The products of this reaction were used to transform competent DH5α E coli, and the bacterial colonies derived from this transformation were used for plasmid mini-preparations.

A total of 10 ampicillin resistant bacterial colonies were used to produce plasmid mini-preparations. A 3 μl aliquot of each plasmid DNA mini-preparation was then digested with *EcoRI* and *XhoI*. The results of this digest are shown in Figure 4.11. Of the 10 digests, 7 reveal the presence of a 1.7 kb insert representing the $G\alpha_{i-3}$ cDNA. Therefore the reference colony corresponding to plasmid preparation number 3 was grown up and used to make a plasmid maxi-preparation. A 5μl aliquot of this plasmid DNA was then digested with *EcoRI* and *XhoI* to ensure that it contained the $G\alpha_{i-3}$ cDNA insert. The results of this digest are shown in Figure 4.12 and demonstrate that the DNA subcloning described above had enabled the construction of the eukaryotic expression pcDNA/$G\alpha_{i-3}$, with the $G\alpha_{i-3}$ insert present at the *EcoRI/XhoI* site of the multiple cloning region of pcDNA3.

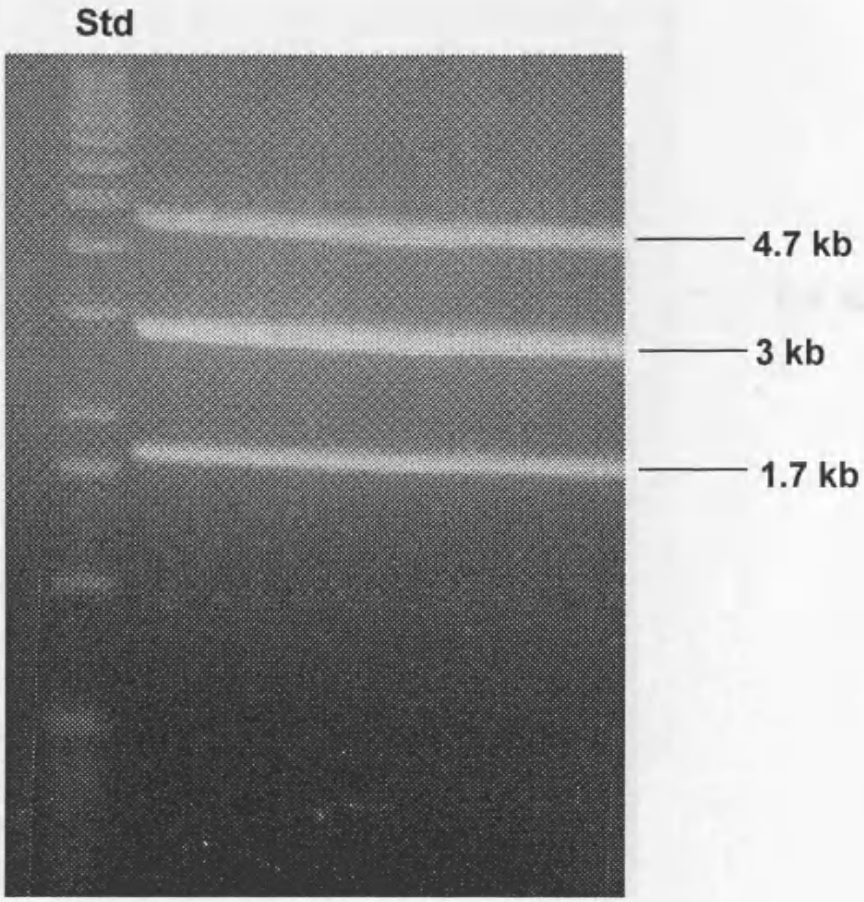


Figure 4.9
Ethidium Bromide Agarose Gel Electrophoresis of pBSII SK/Gα_{i-3} Digested with *EcoRI/XhoI*

10 μg of plasmid pBSII SK/Gα_{i-3} were digested with restriction endonucleases *EcoRI* and *XhoI,* and the resulting DNA fragments separated by ethidium bromide agarose gel electrophoresis, exposed to UV light and photographed. 3 bands of DNA are seen as a result of this digest. The 4.7 kb band represents undigested pBSII SK/Gα_{i-3}, the 3.0 kb band represents linearised pBSII SK from which the Gα_{i-3} cDNA has been excised, and the 1.7 kb band represents the excised Gα_{i-3} cDNA. The 1.7 kb band was cut out, the DNA electroeluted and purified, and then used to ligate into *EcoRI/XhoI* digested pcDNA3

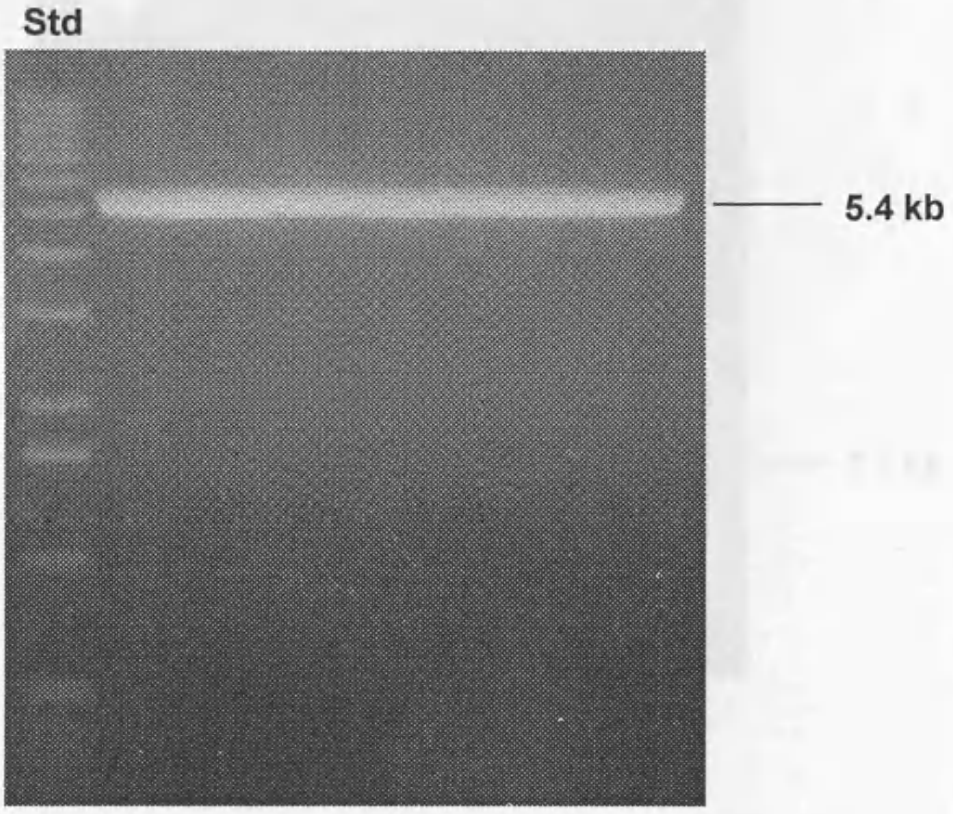


Figure 4.10
Ethidium Bromide Agarose Gel Electrophoresis of Linearised pcDNA3 Digested with *EcoRI* and *XhoI*

10 μg of plasmid pcDNA3 were digested with *EcoRI* and *XhoI* restriction endonuleases. The linearised plasmid of ~5.4 kb was then separated from the tiny *EcoRI/XhoI* excised fragment by ethidium bromide agarose gel electrophoresis, exposed to UV light and photographed. This 5.4 kb fragment of pcDNA3 was then electroeluted and purified. This purified pcDNA3 *EcoRI/XhoI* digest was then used in a ligation reaction with *EcoRI/XhoI* digested $G\alpha_{i\text{-}3}$ cDNA.

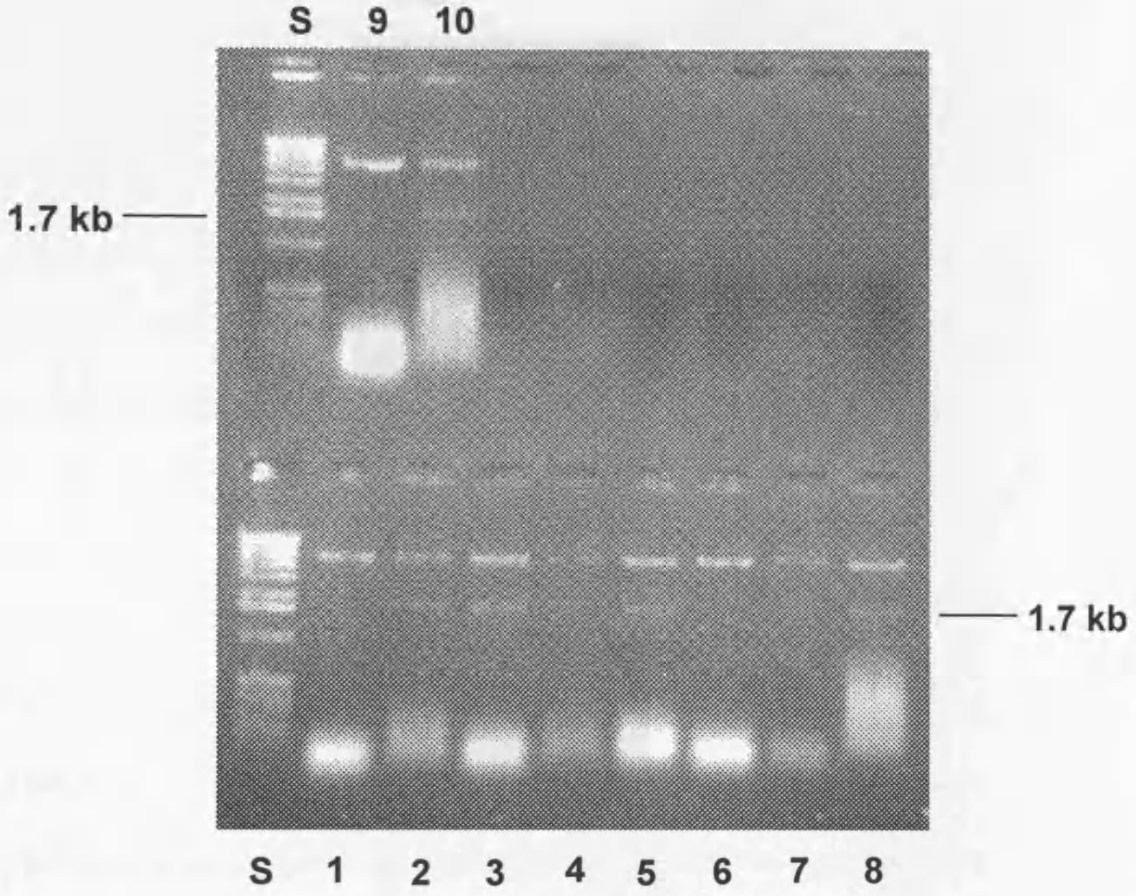


Figure 4.11
Ethidium Bromide Agarose Gel Electrophoresis of pcDNA3/Gα_{i-3} Plasmid Mini-Preps Digested with *EcoRI/Xho1*

Plasmid mini-preps were prepared from10 individual penicillin resistant colonies of DH5α previously transformed with plasmid vector construct pcDNA3/Gα_{i-3}. A 3 μl aliquot of each of these preparations was then digested with the restriction endonucleases *EcoRI* and *XhoI*. The resulting digested DNA fragments were separated by ethidium bromide agarose gel electrophoresis, exposed to UV light and photographed. Lane S = DNA ladder standards. Of the plasmids prepared from the 10 bacterial colonies 7 contain a 1.7 kb *EcoRI/XhoI* insert corresponding to Gα_{i-3} cDNA. The bacterial colony from which the plasmid digest in lane 3 originated was used for larger scale preparation of pcDNA3/Gα_{i-3} for OK cell transfection.

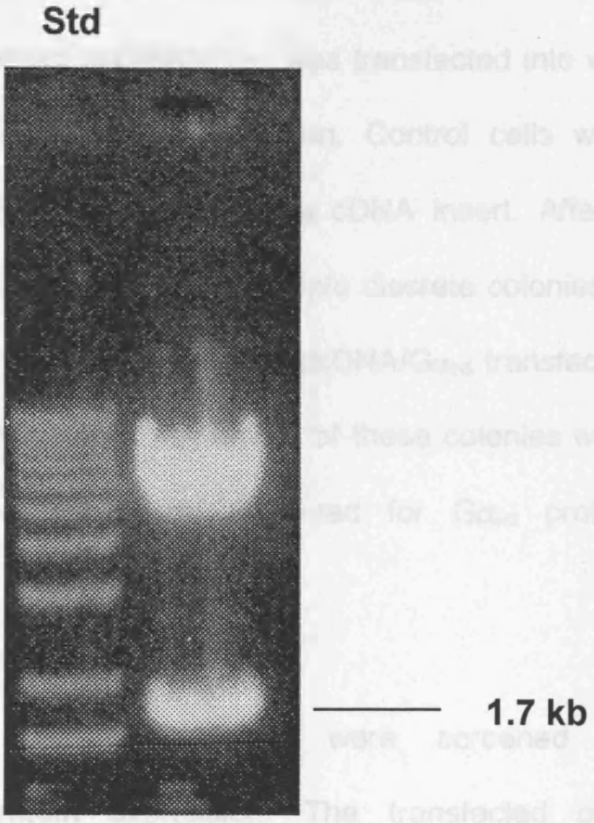


Figure 4.12
Test Digest of Final pcDNA3/Gα_{i-3} Vector Construct Prior to Transfection into OK Cells

The bacterial colony which yielded plasmid mini prep number 3 (Figure 4.11 Lane 3) was expanded and used for a plasmid maxi prep. 5 µl of the plasmid DNA obtained was then digested with restriction endonucleases *EcoRI* and *XhoI*. The DNA fragments obtained in this digestion reaction were separated by ethidium bromide agarose gel electrophoresis, exposed to UV light and photographed. This digest confirms that the pcDNA3/Gα_{i-3} vector construct contains a 1.7 kb insert corresponding to Gα_{i-3} cDNA. Plasmid DNA derived from this preparation was then used to transfect wild type OK cells.

4.7.2 *Transfection of OK cells with pcDNA3/$G\alpha_{i-3}$*

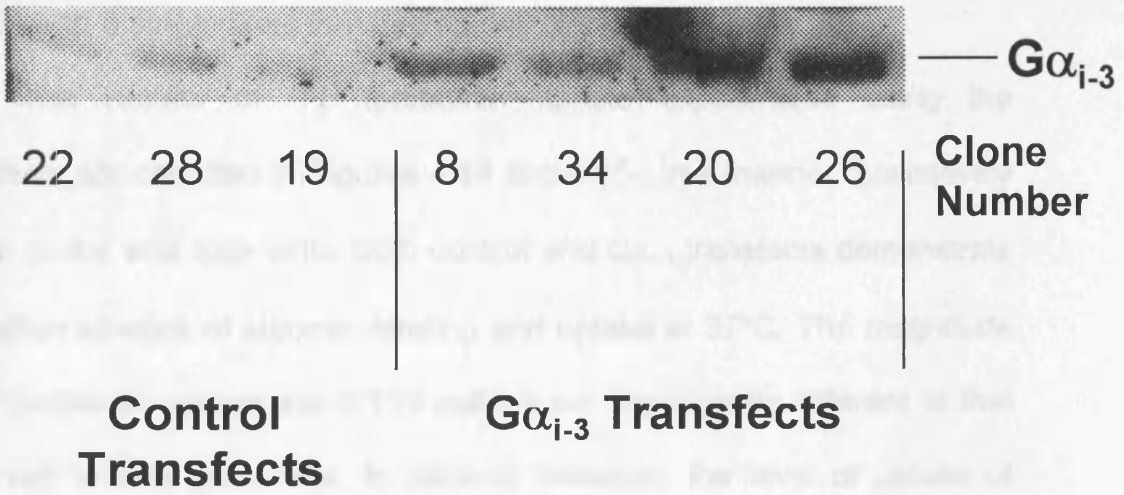
The plasmid DNA construct pcDNA3/$G\alpha_{i-3}$ was transfected into wild type OK cells by calcium phosphate precipitation. Control cells were transfected with pcDNA3 alone lacking the $G\alpha_{i-3}$ cDNA insert. After 5 weeks of growth in G418 containing medium, multiple discrete colonies of G418 resistant OK cells were growing in both the pcDNA/$G\alpha_{i-3}$ transfected and pcDNA3 transfected control plates. A number of these colonies were picked from each plate, expanded and screened for $G\alpha_{i-3}$ protein expression.

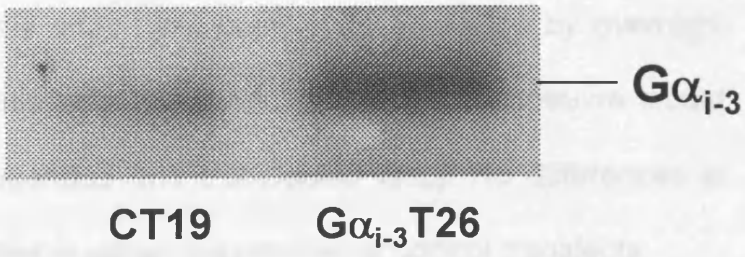
4.7.3 *Screening of transfected cells*

Multiple transfected OK cell clones were screened by immunoblotting for $G\alpha_{i-3}$ protein expression. The transfected cells proliferated and grew normally, and were morphologically indistinguishable from wild type OK cells. All control transfected cells demonstrated a similar level of $G\alpha_{i-3}$ protein expression. Cells transfected with pcDNA/$G\alpha_{i-3}$ demonstrated varying increases in $G\alpha_{i-3}$ protein expression over and above that seen in control transfected cells. Figure 4.13 depicts immunoblots of screened control and $G\alpha_{i-3}$ transfected cells. Control transfected OK cell clone number 19 (CT19) was directly compared to $G\alpha_{i-3}$ transfected OK cell clone number 26 ($G\alpha_{i-3}$T26) for levels of $G\alpha_{i-3}$ protein expression. The $G\alpha_{i-3}$ transfected cell line $G\alpha_{i-3}$T26 expresses a 3.5 fold increase in $G\alpha_{i-3}$ protein, as determined by laser densitometry, compared to the control transfected clone CT19. These two transfected cell lines were used for all the subsequent [^{125}I]-albumin uptake experiments.

Figure 4.13
Immunoblots Depicting Screening of Transfected OK Cells

OK cells were transfected with pcDNA3 or pcDNA3/$G\alpha_{i-3}$ by calcium phosphate precipitation, and cells selected in G418 containing media. Membranes were prepared from a number of both control and $G\alpha_{i-3}$ transfected G418 resistant clones. Transfected cells were screened by immunoblotting of membranes using anti-$G\alpha_{i-3}$ antibodies. Second antibodies were peroxidase conjugated. Blots were developed using an enhanced chemoluminescence system, followed by exposure to photographic film. Equal amounts of membrane protein were applied to each lane. The upper panel depicts the comparison of the $G\alpha_{i-3}$ content of cell membranes derived from a number of control and $G\alpha_{i-3}$ transfected cell clones. Control clones all possess very similar levels of $G\alpha_{i-3}$ protein. $G\alpha_{i-3}$ transfected cells contain variably larger quantities of this protein. The lower panel directly compares levels of $G\alpha_{i-3}$ between control cell clone number 19 (CT19) and $G\alpha_{i-3}$ transfected cell clone number 26 ($G\alpha_{i-3}$T26). These two clonal cell lines were used in the [^{125}I]-albumin uptake experiments. $G\alpha_{i-3}$T26 cells contain 3.5 x higher levels of $G\alpha_{i-3}$ protein than do CT19 cells.


Gαi-3
22
28
19
8
34
20
26
Clone Number
Control Transfects
Gαi-3 Transfects


Gαi-3
CT19
Gαi-3T26

4.8 [^{125}I]-Albumin uptake into transfected OK cells

The rates of albumin uptake in these transfected cells was compared to that of control transfected cells containing the plasmid vector pcDNA3 without the $G\alpha_{i-3}$ cDNA insert. The results presented in this thesis represent the comparison of a single control transfected cell population (CT19) with a single $G\alpha_{i-3}$ transfected cell population ($G\alpha_{i-3}$T26).

The results of [^{125}I]-albumin uptake experiments using the transfects are depicted in Figures 4.14 and 4.15 . In a manner qualitatively similar to the wild type cells, both control and $G\alpha_{i-3}$ transfects demonstrate saturation kinetics of albumin binding and uptake at 37°C. The magnitude of [^{125}I]-albumin uptake into CT19 cells is not significantly different to that observed in wild type cells. In contrast however, the level of uptake of [^{125}I]-albumin into $G\alpha_{i-3}$T26 cells is markedly and significantly increased above that observed with CT19 cells at all incubated concentrations of [^{125}I]-albumin. This observation is due to an increase in the V_{max} of the transport process. The K_M for uptake is unaffected, indicating that the affinity of the transport process for albumin is not changed by modulation of G-protein function. This effect was completely abolished by overnight pre-incubation of the cells with pertussis toxin. This manoeuvre would serve to inactivate endogenous and transfected $G\alpha_{i-3}$. No differences in albumin binding were noted in either the positive or control transfects.

To ascertain whether $G\alpha_{i-3}$ regulated an early or late step in the endocytic pathway we examined the time course of [^{125}I]-albumin uptake into transfected cells. The time course of [^{125}I]-albumin uptake into CT19

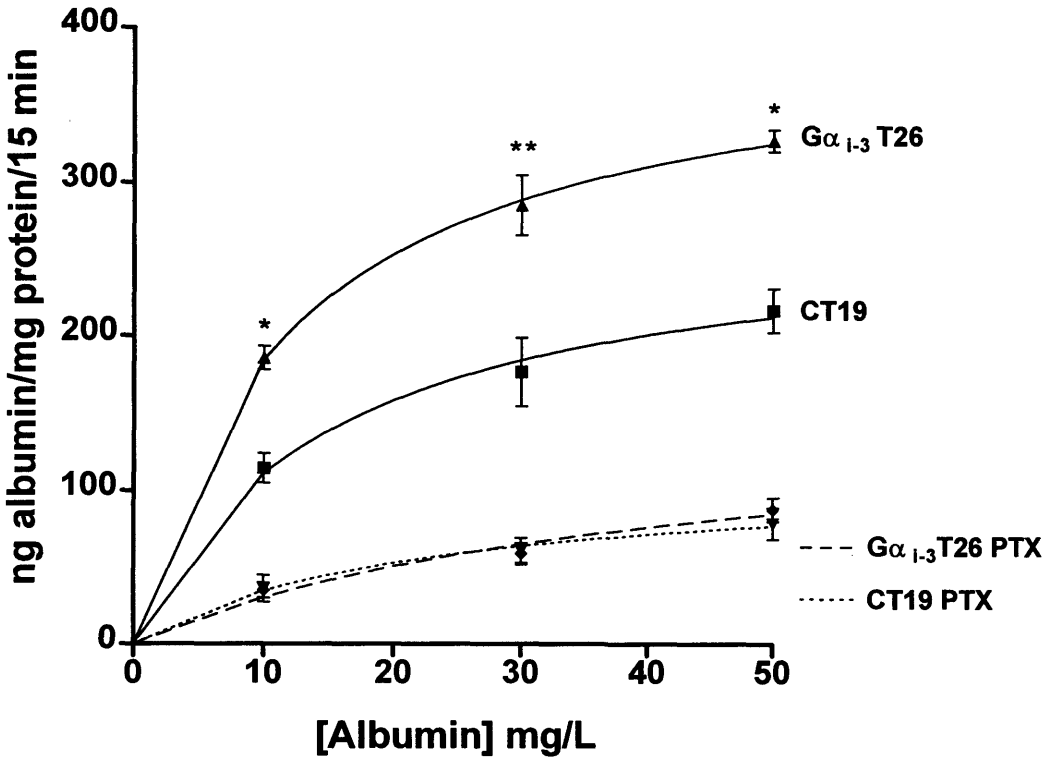


Figure 4.14
Concentration Dependency of [^{125}I]-Albumin Uptake into Transfected OK Cells

Albumin uptake into both CT19 and G α_{i-3}T26 transfected OK cells was measured using various concentrations of [^{125}I]-albumin for 15 minutes at 37°C. These curves represent the lower range of [^{125}I]-albumin concentrations used under control conditions (solid lines), and after pre-treatment with pertussis toxin (broken lines). Gα_{i-3}T26 transfects take up significantly greater amounts of [^{125}I]- albumin at all incubated concentrations than do CT19 transfected cells. This effect is completely reversed by pre-incubation with pertussis toxin (long dashed line, Gα_{i-3}T26; short dashed line CT19). Values are means ± SE of at least 3 experiments performed in duplicate. * $p < 0.01$, ** $p < 0.05$.

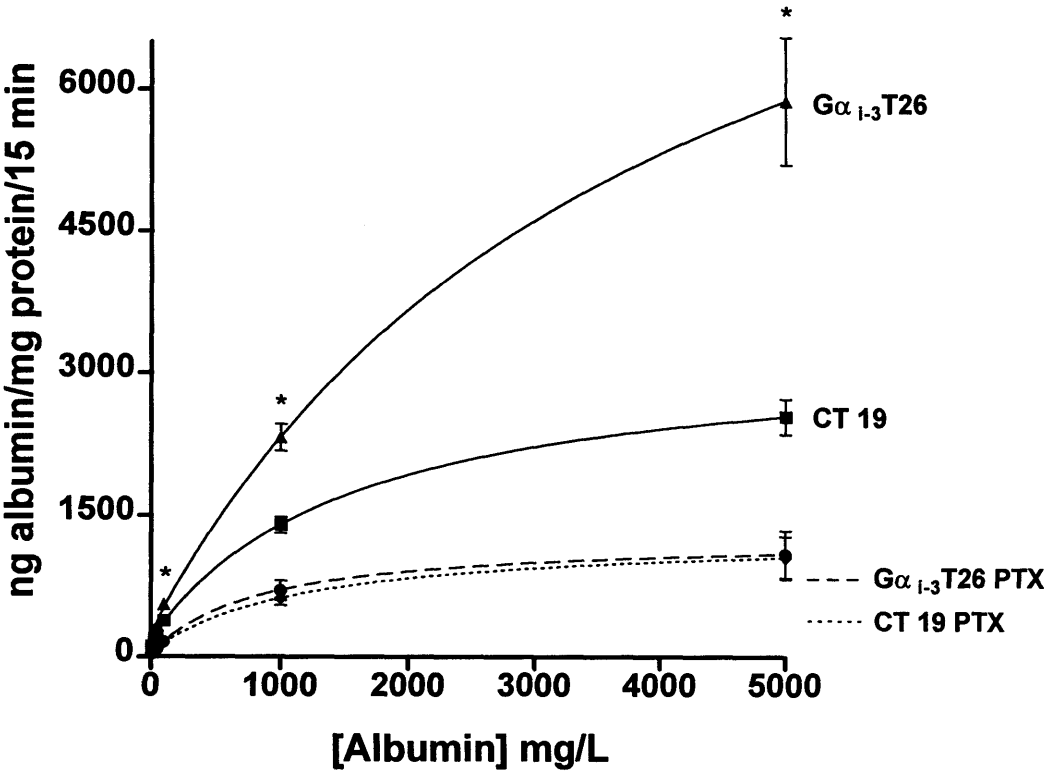


Figure 4.15
Concentration Dependency of [^{125}I]-Albumin Uptake into Transfected OK Cells

Albumin uptake into both CT19 and $G\alpha_{i\text{-}3}T26$ transfected OK cells was measured using various concentrations of [^{125}I]-albumin for 15 minutes at 37°C. These curves represent the full range of [^{125}I]-albumin concentrations used under control conditions (solid lines) and after pre-treatment with pertussis toxin (broken lines). G $\alpha_{i\text{-}3}T26$ transfects take up significantly greater amounts of [^{125}I]- albumin at all incubated concentrations than do CT19 transfected cells. This effect is completely reversed by pre-incubation with pertussis toxin (long dashed line, $G\alpha_{i\text{-}3}T26$; short dashed line CT19). Values are means ± SE of at least 3 experiments performed in duplicate. * $p < 0.01$.

cells demonstrates a rapid initial rise reaching a peak after 30 minutes. This is then followed by a plateau. These results are depicted in Figure 4.16. The most striking differences between the two transfected cell populations are apparent at the earliest time point, with the majority of the increase in albumin uptake seen in the $G\alpha_{i-3}T26$ cells being observed after 5 minutes. The peak of [^{125}I]-albumin uptake is still observed at 30 minutes, but is considerably enhanced in magnitude in the $G\alpha_{i-3}T26$ cells compared to CT19 cells. In a similar pattern to the [^{125}I]-albumin uptake seen in CT 19 cells, the peak is followed by a plateau. These observations suggest therefore that the G-protein, $G\alpha_{i-3}$, regulates an early step in the endocytosis of [^{125}I]-albumin by OK cells.

4.9 Endocytosis of [^{125}I]-albumin after pre-treatment of wild type OK cells with phorbol esters

In order to activate PKC enzymes wild type OK cells were pre-treated with the phorbol ester PDBU as described in section **2.15**. The concentration dependency of albumin uptake by the high affinity system (described in **4.1**) into these PDBU pre-treated cells is depicted in Figure 4.17. It can be seen that pre-treatment of the cells with PDBU had no significant effect on [^{125}I]-albumin uptake into wild type OK cells any incubated concentration of [^{125}I]-albumin. The [^{125}I]-albumin saturation uptake curves for control untreated cells and PDBU pre-treated cells are almost identical. Analysis of this experimental data by non-linear curve fitting reveals that the K_M for this process in control cells is 26.7 ± 7.8 mg/L, with a V_{max} of 464.1 ± 48.75 ng/mg protein. In the cells pre-treated with

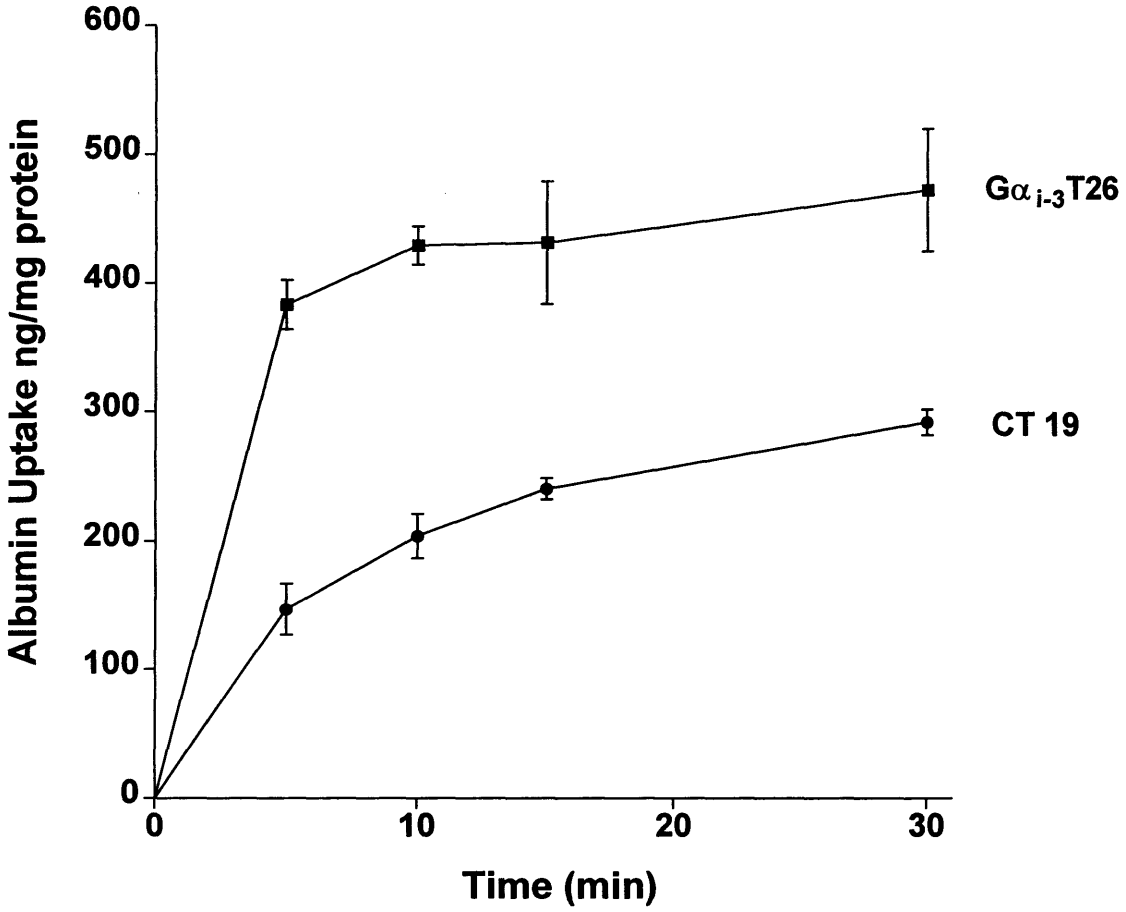


Figure 4.16
Time Course of Uptake of [^{125}I]-Albumin into Transfected OK Cells

CT19 (●) and $G\alpha_{i\text{-}3}T26$ (■) transfected OK cells were grown to confluence in 6 well plates. Cells were incubated with 100 mg/L [^{125}I]-albumin for various times at 37°C. Values for both non-specific and specific albumin binding, as measured at 4°C for each time point, have been subtracted from this data. Values represent means ± SE, n = ≥3 experiments performed in duplicate.

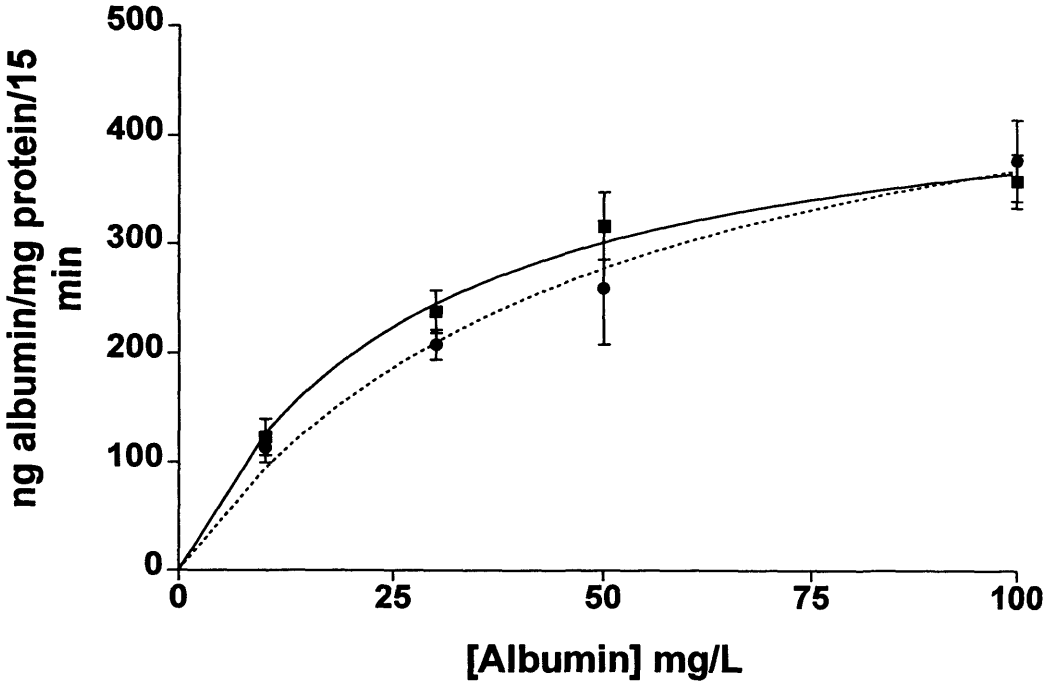


Figure 4.17
Effect of Phorbol Esters on Albumin Uptake into Wild Type OK Cells

[^{125}I]-albumin uptake was measured in wild type OK cells after 15 minutes of incubation at 37°C under control conditions (■) or after pre-treatment with PDBU (●, dashed line). Values for non-specific binding of albumin have been subtracted. Values are means ± SE of 3 experiments performed in duplicate.

PDBU the K_M is 48.0 ± 21.8 mg/L and the V_{max} 545.5 ± 113.3 ng/mg protein. Hence PKC activation appears to have no effect on either the affinity of the uptake process for albumin, or the V_{max} of albumin uptake by this endocytic system.

4.10 Endocytosis of [^{125}I]-albumin by wild type OK cells pre-treated with butanol

Wild type OK cells were pre-treated with butanol in order to inhibit the activity of the enzyme PLD (section **2.16**). Figure 4.18 shows the effect of pre-treating wild type OK cells with increasing concentrations of butanol on subsequent [^{125}I]-albumin endocytosis. The concentrations of butanol used to pre-treat the cells ranged from 1 μM to 50 mM. It can be clearly seen that no significant alteration in [^{125}I]-albumin uptake occurs as a consequence of cell pre-incubation with any concentration of butanol.

4.11 Discussion

The results of these experiments confirm the previous report of Schwegler *et al* (1991) which documented the receptor mediated endocytosis of albumin in OK cells. Furthermore the present data provide the first evidence that this is a regulated process, and indeed that heterotrimeric G-proteins are involved in the regulation of receptor mediated endocytosis of protein in the kidney proximal tubular cell. The enhanced [^{125}I]-albumin uptake observed with elevated levels of $G\alpha_{i-3}$ being completely reversed by pertussis toxin pre-treatment confirming that this

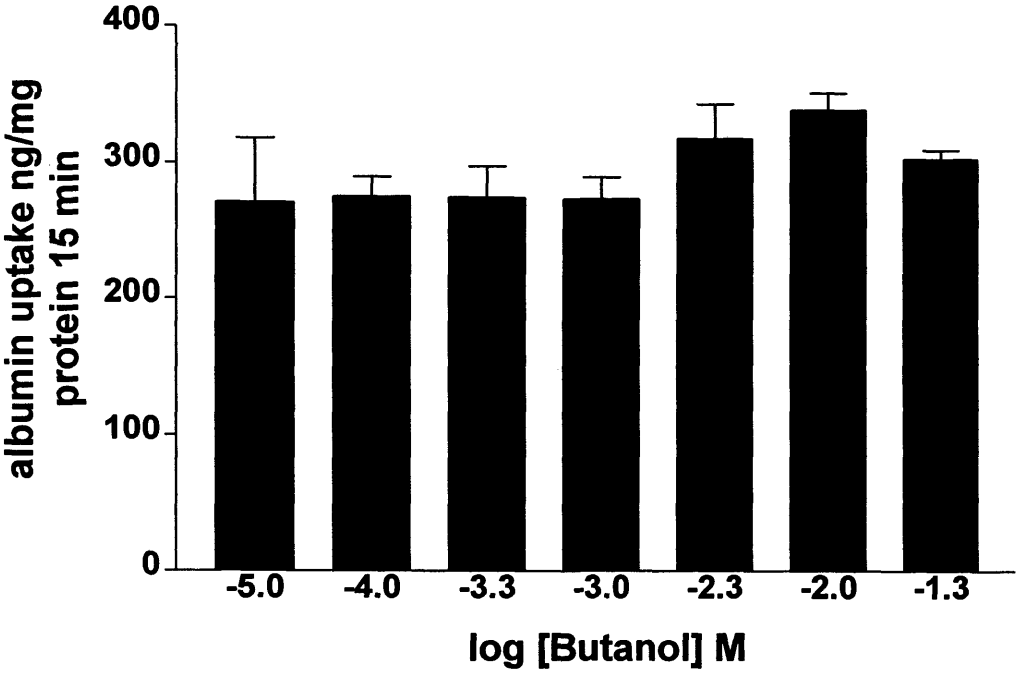


Figure 4.18
Dose Response Effect of Butanol on Albumin Uptake into Wild Type OK Cells

[^{125}I]-albumin uptake was measured in wild type OK cells after pre-treatment with various concentrations of butanol. The concentration of [^{125}I]-albumin used was 150 mg/L in all experiments. Data represent means ± SE of n = 3 experimants performed in duplicate. No significant differences in albumin uptake are observed with any concentration of butanol pre-incubation

G-protein subunit is involved in the regulation of receptor mediated protein endocytosis in this setting.

Although the characteristics of [^{125}I]-albumin binding are broadly in agreement with Schwegler *et al* (1991), some differences do exist. The present results provide evidence for two albumin uptake systems in OK cells whereas the results of Schwegler *et al* (1991) described only one. However the albumin uptake experiments described in this thesis utilised incubated albumin concentrations up to 10000 mg/L, and as such two apparent components of uptake are clearly observed. Using non-linear curve fitting software a high affinity uptake system with apparent K_M 24.3 mg/L and a low affinity uptake system with apparent K_M 15.9 g/L are resolved. Schwegler et al on the other hand employed lower incubated albumin concentrations of up to 300 mg/L, and were able to document only one albumin transport system, according to Michaelis Menton kinetics, with an apparent K_M of 24 mg/L. Clearly this is very much in accordance with the results of the current experiments relating to the higher affinity uptake system. These values can only be regarded as approximate however since albumin uptake measured in these experiments consists of a composite of binding and endocytic uptake by both receptor mediated and fluid phase pathways. The inulin uptake data however suggest that fluid phase endocytosis can account for only a small proportion of total albumin uptake measured in these experiments. Nonetheless the apparent K_M of albumin for the high affinity system is in close approximation to the measured albumin concentration in proximal tubular fluid.

Park and Maack (1984) have examined the kinetics of [^{3}H]-albumin binding to proximal tubules using isolated perfused rabbit proximal tubular segments. In this model, utilising a concentration range of perfused albumin comparable to the current experiments two components of albumin absorption were resolved, a high affinity site with K_M 31 mg/L, and a low affinity site with K_M 1.2 g/L. Again the K_M of this high affinity site is very similar to that described in this current work. Although there is a 10-fold difference between this low affinity site (Park and Maack 1984) and that described in the current experiments, it is difficult to extrapolate the results of albumin uptake in cells with a dense brush border to the results obtained in those cells lacking such a membrane.

The results of the electron microscopy of OK cells incubated with gold-albumin demonstrate binding of gold-albumin in apical membrane invaginations together with the presence of gold-albumin in early intermediate, and late components of the intracellular endocytic pathway. Hence these observations provided confirmation that after cell surface binding gold-albumin entered an intracellular vesicular trafficking pathway.

Despite the bulk of morphological data and the more scanty kinetic data relating to albumin absorption in proximal tubules, little is known of the regulatory mechanisms underpinning receptor mediated endocytosis in this setting. Inhibition of [^{125}I]-albumin endocytosis by NEM is in keeping with uptake into a vesicular trafficking pathway. This widely reactive cysteine alkylating agent inactivates $G\alpha_i$ linked responses at the concentrations used in this study, whilst having little effect on the function

of other G-proteins such as those of the $G\alpha_s$ family (Jakobs *et al* 1982, Smith and Harden 1984, Wong *et al* 1985). However NEM is relatively non-specific in it's action. In fact some protein regulatory components of the vesicular trafficking system are defined according to their sensitivity to NEM (Rothman 1994, Schekman and Orci 1996). Clearly therefore the inhibitory effect of NEM on [^{125}I]-albumin endocytosis may be due to it's effects on any one of a number intracellular targets. Nonetheless the inhibitory effect of NEM on [^{125}I]-albumin endocytosis is in keeping with it's effect on vesicular transport even though the target cannot be precisely identified as a G-protein.

The results provided by the pertussis and cholera toxin experiments can be interpreted much more specifically. These data confirm the involvement of a heterotrimeric G-protein in endocytic regulation. The inhibition of albumin uptake by pertussis toxin suggests a regulatory role for $G\alpha_i$ or $G\alpha_o$ in endocytosis itself, since pre-incubation of the cells with the pertussis toxin has no effect on albumin binding to the OK cells. Furthermore the observed, albeit less marked, reduction in the fluid phase endocytosis of inulin in the presence of pertussis toxin suggests that the G-protein regulates a step(s) in the endocytic pathway downstream of any membrane binding events. The lack of effect of cholera toxin excludes both a $G\alpha_s$ mediated effect, and any non-specific intoxicating effect of ADP-ribosylating bacterial toxins on this cell type.

However it is clear from the time course of [^{125}I]-albumin uptake into the transfects that the regulatory influence of the G-protein is at an early

step in the endocytic pathway. This observation is in keeping with the findings of Carter et al (1993) who described the involvement of several G-proteins in a number of early steps in receptor mediated endocytosis via clathrin coated vesicles (8).

The next step was to determine exactly which of the candidate heterotrimeric G-proteins was responsible for the observed regulation of [^{125}I]-albumin endocytosis in OK cells. Previous studies have examined the distribution of several G-protein subunits in the kidney (Brunskill *et al* 1991, Stow *et al* 1991b). Surprisingly large quantities of G-proteins appear to be localised to the apical membrane of kidney tubules, a region not noted as a site of major hormone signalling, thus suggesting that these G-proteins may have novel functions. In particular, appreciable amounts of $G\alpha_{i-3}$ and $G\alpha_s$ are observed to be present in this membrane domain. In contrast much smaller amounts of $G\alpha_{i-2}$ are seen in this region, and $G\alpha_{i-1}$ is not detected at all by immunocytochemistry (Stow *et al* 1991b). Furthermore $G\alpha_o$ is not detected in kidney cortex by immunoblotting (Murakami *et al* 1989). Therefore the observation of large quantities of $G\alpha_{i-3}$ protein in kidney proximal tubule apical membranes, together with the absence of a significant cholera toxin effect in the current experiments, and the small quantities/absence of $G\alpha_{i-1}$, $G\alpha_{i-2}$, and $G\alpha_o$ in this membrane, made the $G\alpha_{i-3}$ protein in particular an attractive candidate for further study.

Therefore the cDNA for this protein was transfected into OK cells and overexpressing transfects generated. Significant enhancement of

albumin uptake by $G\alpha_{i-3}$ overexpression, which is reversed by pertussis toxin pre-treatment, strongly suggests that the G_i family member responsible for the observed effect is $G\alpha_{i-3}$. The results of the experiments strongly suggest that the G-protein directly regulates the rate of endocytic transport of protein, rather that having an effect on the affinity of the transport process for the albumin itself. The time course studies of [^{125}I]-albumin uptake into the transfects indicates that the regulatory influence of the G-protein is at an early step in the endocytic pathway. This observation is also in keeping with the description by Carter et al (1993) of the involvement of several G-proteins in numerous early steps in receptor mediated endocytosis via clathrin coated vesicles.

The data presented in this thesis, taken together with that of other authors discussed earlier, strongly supports a critical role for heterotrimeric G-proteins in the regulation of vesicular transport. It is currently not clear how G-proteins may function to regulate receptor mediated endocytosis of albumin. The widely accepted paradigm of G-protein action dictates that an upstream receptor links through the G-protein to a downstream effector. The inhibitory effect of pertussis toxin on the function of $G\alpha_{i-3}$ in the regulation of endocytosis is consistent with this orthodox G-protein model, but the identities of the receptor and effector components of the system are not addressed by this paper and their nature remains obscure. Whether agonists which activate $G\alpha_{i-3}$ through classical seven trans-membrane spanning receptors also activate endocytic pathways is unknown.

Having established that receptor mediated endocytosis of albumin in OK cells is regulated by heterotrimeric G-proteins the next step was to try and determine the identities of other enzymes involved in the regulation of this process. As discussed previously in section **1.5.4** two enzymes implicated in vesicular protein trafficking in other systems are PKC and PLD. Well established protocols were therefore employed to stimulate PKC using the phorbol ester PDBU (Ryves *et al* 1991), inhibit PLD using butanol (Boarder and Purkiss 1993). However the results of these experiments are clear cut and support the conclusion that neither PKC nor PLD appear to play a significant role in the regulation of receptor mediated endocytosis of albumin by OK cells. Nonetheless the evidence that protein phosphorylation/de-phosphorylation has an important regulatory role in endocytic pathways is strong, and it must be stressed that these results do not exclude an important role of phosphatases, or other kinases not examined in this thesis, in the control of endocytosis.

Likewise the results of the butanol pre-treatment experiments indicate that PLD is not significantly involved in the control of albumin endocytosis by OK cells. The potential for a major role of other phospholipases in this process has not been addressed by the experiments described, and as such cannot be excluded.

Importantly however the results documented in this thesis give some insight into the means of regulation of proximal tubular albumin endocytosis. The next challenge is to manipulate this process in order to

abrogate the potential pathophysiological effects of albumin absorption on proximal tubule cell and whole kidney function.

Chapter 5

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

This investigation set out to examine the interaction of albumin with proximal tubular epithelial cells. The driving force for the work was a number of clinical observations implicating filtered proteins, particularly albumin, in the renal scarring and dysfunction observed in patients with proteinuria. Major aims of the work were to establish whether specific receptors for albumin exist in the proximal tubule, to establish the nature and characteristics of any potential receptors, to determine whether the endocytosis of albumin, occurring subsequent to binding, was regulated by heterotrimeric G-proteins, and to assess whether two important intracellular enzymes, PKC and PLD, were able to regulate the albumin endocytic activity of OK cells.

As a result of this work novel receptors for albumin have been identified in proximal tubular epithelial cells. Binding of albumin to these receptors occurs within the physiological range to be expected given the concentration of albumin found in the glomerular filtrate. These receptors are apparently homologues of albumin binding proteins found in other cell types, notably endothelial cells, and conform to the ligand binding profile of scavenger receptors (Krieger and Herz 1994). Other workers have also investigated the nature of the albumin receptor in the proximal tubule of the kidney. The results of these investigators, published

contemporaneously with the experiments documented in this thesis, provide some support for the results in this thesis. Cessac-Guillemet *et al* (1996) used a different method to identify albumin binding proteins in rat kidney brush border preparations. A major albumin binding protein of 31 kDa was identified together with a further protein of 55 kDa. This 31 kDa protein is likely to be the same protein identified at 30 kDa by [^{125}I]-albumin ligand blotting as described in this thesis. The nature of the 55 kDa protein identified by Cessac-Guillemet *et al* (1996) is unclear, but it's apparent molecular weight is similar to that of albondin (Schnitzer 1992).

Other candidate receptors for albumin in the kidney proximal tubule have been proposed. Recently published work by Cui *et al* (1996) has suggested that albumin may bind to megalin/gp330 in rat proximal tubules. This large protein, one of the Heymann nephritis antigens (Kerjaschki and Farquhar *et al* 1982), is a member of the low density lipoprotein receptor gene family (Saito *et al* 1994), is widely distributed in the proximal tubule (Christensen *et al* 1995) and has a broad specificity for a number of protein ligands (Moestrup *et al* 1994). The precise relationship between gp330/megalin and gp14, gp18, gp30, and gp60 is unclear. It is conceivable that the lower molecular weight albumin binding proteins are breakdown products of gp330/megalin. However the expression of gp330/megalin is highly restricted to specialised absorptive epithelia (Kounnas *et al* 1994), whereas the gp14, gp18, gp30 and gp60 appear to be more widely distributed as discussed earlier, and it therefore seems unlikely that they could represent fragments of gp330/megalin.

Nonetheless it is possible that some of these glycoproteins may represent individual subunits of a single receptor complex. It is also worth reiterating that the the calculated affinities of albumin binding to both gp30 and gp18 in the ligand blot experiments described in this thesis are very similar. Also these affinities are very similar to the K_D of the higher affinity binding site for albumin in intact OK cell monolayers calculated from the experimental data presented in this thesis. These observations suggest firstly that gp18 may be a truncated form of gp30, and secondly that these albumin binding protein(s) are likely to comprise the higher affinity albumin binding site detected in intact cells. The identity of the lower affinity binding site is not provided by these ligand blotting experiments.

It is evident that considerable work is yet required to determine the exact nature of the albumin receptors in the proximal tubule, and the relationships between them. The lectin inhibition experiments described in this thesis suggest an approach to purifying albumin binding proteins by lectin affinity chromatography. Such an approach may eventually allow the purification of sufficient quantities of these albumin binding proteins to allow partial amino acid sequencing and then ultimately DNA sequencing of these polypeptides.

The confirmation of heterotrimeric G-protein regulation of endocytic transport illustrates the burgeoning importance of these proteins in this area of cell biology, but also poses a number of interesting and important questions. The classical signal transduction cascade described in **1.5.3** operates at the plasma membrane, whereas many steps in endocytic

transport are intracellular and not directly related to the cell membrane. Bearing this in mind therefore it is necessary to ask whether the mechanism of heterotrimeric G-protein activation and regulation of albumin endocytosis conforms to this widely accepted paradigm. To address this question it is useful to consider again the effect of pertussis toxin on endocytosis demonstrated in this thesis. Pertussis toxin catalyses ADP ribosylation of a cysteine residue within the C-terminus of $G\alpha_i$. The effect of this covalent modification of the α-subunit is to interfere with the binding of the α-subunit to it's receptor and thus prevent activation of the heterotrimeric G-protein (Moss and Vaughan 1990). Clearly therefore the inhibitory effect of pertussis toxin on albumin endocytosis is consistent with a heterotrimeric G-protein acting according to the orthodox model. Other questions arise naturally from this conclusion. For example what is the receptor(s) which activates the heterotrimeric G-protein regulating endocytosis, and is it extra- or intracellular? The answers to this question are completely unknown, but the future identification of receptors and their ligands which may control the rate of endocytosis could potentially provide opportunities for pharmacological intervention. Some authors have postulated that G-proteins may control endocytosis in a manner dissimilar to that suggested by the classical model (Helms 1995). These proposals are controversial, but future work should reveal interesting insights into this important question.

Therefore the results in this thesis demonstrate that the endocytosis of albumin by the OK cell is subject to regulatory input, and as such it may,

in the fullness of time, be possible to down-regulate the albumin uptake pathway with the potential for abrogating renal scarring. Certainly there is much left to learn about this pathway, for instance does the stimulation of GTP/GDP exchange on $G\alpha_{i\text{-}3}$ in the classical manner by ligand occupied (as yet unidentified) plasma membrane receptors, also stimulate the endocytic pathway?

Evidence supports the involvement of numerous enzymes and signalling pathways in the regulation of albumin endocytosis. Inhibition of albumin endocytosis is observed during elevations of cAMP in opossum kidney cells. The process is also dependent on Ca^{2+} ions (Gekle *et al* 1995). There is little doubt that kinases and phosphatases will ultimately prove to be important in the regulation of endocytosis and exocytosis (discussed in section **1.5.4**). It was somewhat surprising to find that modulation of both PKC and PLD activity, by phorbol esters and butanol respectively, had no effect on the receptor mediated endocytosis of albumin in OK cells. This observation does not imply that these enzymes lack any role in vesicular transport in other systems or other cells, but certainly suggests that they do not have general importance in all vesicular transport events.

Future efforts will be directed towards further elucidation of the role of kinase/phosphatase systems in endocytic regulation using other specific and non-specific inhibitors of these enzymes. In addition attention will be given to other enzymes, in particular the lipid kinases such as the phosphoinositide 3-kinases (Vanhaesebroeck *et al* 1997), which along with

$G\alpha_{i-3}$, may be able to regulate the rate of albumin endocytosis in the proximal tubule.

Chapter 6

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