The EMBO Journal Vol.16 No.1 pp.35–43, 1997

Ezrin is a cyclic AMP-dependent protein kinase anchoring protein

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cAMP-dependent protein kinase (A-kinase) anchoring proteins (AKAPs) are responsible for the subcellular sequestration of the type II A-kinase. Previously, we identified a 78 kDa AKAP which was enriched in gastric parietal cells. We have now purified the 78 kDa AKAP to homogeneity from gastric fundic mucosal supernates using type II A-kinase regulatory subunit (R_{II}) affinity chromatography. The purified 78 kDa AKAP was recognized by monoclonal antibodies against ezrin, the canalicular actin-associated protein. Recombinant ezrin produced in either Sf9 cells or bacteria also bound R_{II}. Recombinant radixin and moesin, ezrin-related proteins, also bound R_{II} in blot overlay. Analysis of recombinant truncations of ezrin mapped the R_{II} binding site to a region between amino acids 373 and 439. This region contained a 14-amino-acid amphipathic α-helical putative R_{II} binding region. A synthetic peptide containing the amphipathic helical region ($ezrin_{409–438}$) blocked R_{II} binding to ezrin, but a peptide with a leucine to proline substitution at amino acid 421 failed to inhibit R_{II} binding. In mouse fundic mucosa, R_{II} immunoreactivity redistributed from a predominantly cytosolic location in resting parietal cells, to a canalicular pattern in mucosa from animals stimulated with gastrin. These results demonstrate that ezrin is a major AKAP in gastric parietal cells and may function to tether type II A-kinase to a region near the secretory canaliculus.

Keywords: anchoring protein/cAMP-dependent kinase/ezrin/gastric parietal cells

Introduction

Epithelial cells, by definition, segregate the components of their apical and basolateral domains in order to express and maintain specialized functions at disparate surface environments. The proper segregation of both second messenger systems and specialized membrane–cytoskeleton complexes contributes, among other factors, to epithelial integrity and function. In the gastric parietal cell, histamine stimulates an increase in cAMP, leading to a marked morphological rearrangement, which elicits the fusion of H/K-ATPase-containing tubulovesicles with the intracellular canalicular target membrane (Forte *et al.*, 1977; Soroka *et al.*, 1993). These structural changes represent one of the largest reversible membrane–cytoskeletal rearrangements in eukaryotic cells. Several investigations have demonstrated the histamine-induced phosphorylation of both cytosolic and particulate substrates in gastric parietal cells (Chew and Brown, 1987; Urushidani *et al.*, 1987, 1989; Oddsdottir *et al.*, 1988). The only well-characterized histamine-stimulated phosphoprotein is ezrin, which co-localizes with both F-actin filaments and the intracellular canalicular membranes (Hanzel *et al.*, 1989, 1991). In this cell system, as in other epithelial cells, ezrin is thought to play a major role as both a membrane–cytoskeletal linker (Hanzel *et al.*, 1991; Algrain *et al.*, 1993) and a critical component in microvilli biogenesis (Takeuchi, *et al.*, 1994; Berryman *et al.*, 1995).

While both type I and type II cAMP-dependent protein kinases (A-kinases) are present in gastric parietal cells, the type I A-kinase is predominantly cytosolic, while the type II enzyme partitions into both cytosolic and particulate fractions (Corbin *et al.*, 1977; Chew, 1985; Goldenring *et al.*, 1992). Chew (1985) has demonstrated that elevation of intracellular cAMP activates cytosolic type I A-kinase. In addition, we have observed that stimulation of parietal cells with histamine also induces dephosphorylation of the particulate regulatory subunit of type II A-kinase (R_{II}) (Goldenring *et al.*, 1992). The dephosphorylation of R_{II} recently has been correlated with dissociation of the type II holoenzyme and kinase activation (Dibona *et al.*, 1979). Together, these data suggested that subcellular activation of A-kinase in parietal cells might be regulated through sequestration by specific A-kinase anchoring proteins (AKAPs) (Scott and Carr, 1992). More recently, we have characterized a 78 kDa AKAP (AKAP78) which was enriched significantly in gastric parietal cells (Dransfield *et al.*, 1995). This AKAP distributed predominantly into a Triton X-100-insoluble fraction (Dransfield *et al.*, 1995), suggesting its association with the cytoskeleton. We have now sought to isolate and identify AKAP78 from parietal cells. The results indicate that AKAP78 is ezrin, a member of a family of actin-associated proteins.

Results

Isolation of gastric AKAP78

Since AKAP78 was present in both particulate and cytosolic fractions, we purified AKAP78 from 100 000 *g* gastric fundic mucosal supernates using R_{II} affinity chromatography. Recombinant R_{II} was bound to cAMP–agarose resin, and the resulting R_{II} affinity resin was then used to resolve gastric fundic mucosal supernates. Figure 1 demonstrates that the majority of protein voided the R_{II} affinity column. However, most of the AKAP78 was

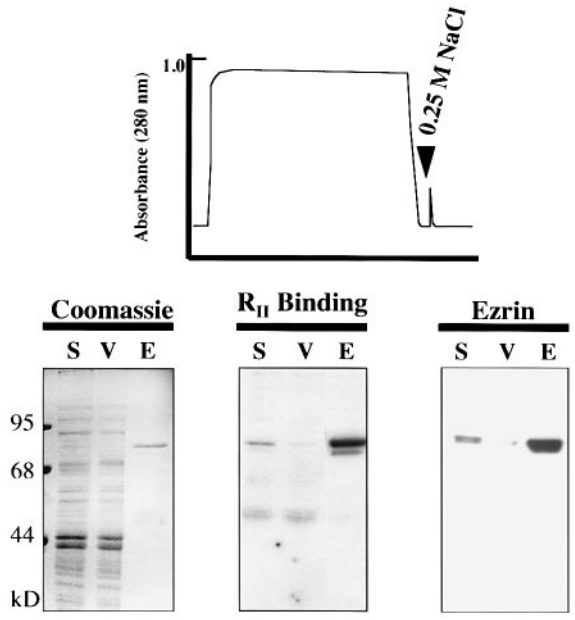

Fig. 1. Purification of gastric AKAP78. Gastric fundic mucosal high speed supernates from two rabbit fundi were chromatographed over R_{II} affinity resin. Upper panel: the majority of protein, as monitored by A_{280}, voided the column. A sharp elution of protein was observed at 250 mM NaCl. No further elutions of protein were detectable at either higher concentrations of NaCl or with 1 mM cAMP (data not shown). Lower panels: Coomassie blue staining demonstrated, in comparison with the unresolved gastric fundic mucosal supernate (S), a decrease in the staining in the void fraction (V) for a 78 kDa protein, which was eluted as an essentially homogeneous fraction with 250 mM NaCl (E). Examination of $[^{32}P]R_{II}$ binding to these three fractions also demonstrated the loss of binding to AKAP78 in the void fraction with elution of this protein in the 250 mM NaCl fraction. A minor 75 kDa breakdown product was observed variably. Examination of the three fractions for ezrin immunoreactivity showed a similar diminution of ezrin immunoreactivity in the void fraction with enrichment into the 250 mM NaCl fraction. The results are representative of three separate experiments.

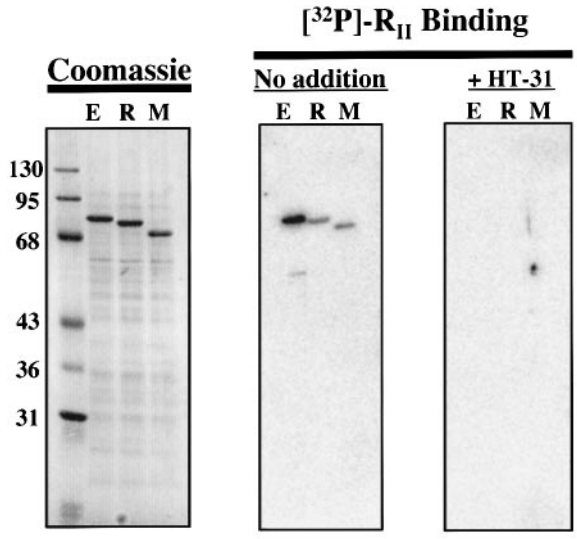

Fig. 2. Binding of $[^{32}P]R_{II}$ to ERM protein constructs. Total extracts of 10 000 Sf9 cells expressing full-length recombinant ezrin (E), radixin (R) and moesin (M) were resolved on 8% SDS–PAGE gels and either stained with Coomassie blue, or transferred to nitrocellulose and probed with $[^{32}P]R_{II}$ in the absence or presence of 1 μM HT-31 peptide. Specific binding to all three ERM proteins that was inhibited in the presence of HT-31 peptide was observed. The results are representative of two separate experiments.

retained on the resin, as evidenced by a decrease in $[^{32}P]R_{II}$ binding to AKAP78 in the void fraction. Examination of the eluted fraction on Coomassie blue-stained gels revealed an essentially homogeneous fraction containing a single 78 kDa band. A minor band of ~75 kDa, which also bound R_{II}, was observed variably and appears to be a breakdown product of the 78 kDa band. The 250 mM NaCl eluate contained a marked enrichment in AKAP78 R_{II} binding. Further elution of the resin with higher NaCl concentrations up to 1.0 M failed to elute any other R_{II} binding proteins, and no significant protein elution was observed either by A_{280} or Coomassie blue staining. Additional elution of the column overnight with 10 mM cAMP resulted in the elution of recombinant R_{II} (data not shown), but no further AKAPs were co-eluted. In contrast to the results obtained with the R_{II} affinity column, chromatography of gastric supernate over cAMP resin retained $<5\%$ of the cytosolic ezrin (data not shown). Ezrin which was retained on cAMP resin could be eluted with either 250 mM NaCl or 1 mM cAMP. These results suggested that a small population of ezrin is associated with R_{II} in the cytosolic fraction.

Given the prominence of the eluted AKAP78 band, we investigated the possibility that AKAP78 might be the canalicular-associated protein, ezrin (Hanzel *et al.*, 1991). Monoclonal antibodies against ezrin documented the presence of ezrin in the gastric fundic mucosal supernates, with both a decrease in ezrin immunoreactivity in the void fraction of the R_{II} affinity column and an enrichment of ezrin immunoreactivity in the 250 mM NaCl eluate paralleling the enrichment for R_{II} binding (Figure 1). These results suggested that AKAP78 was indeed ezrin.

R_{II} binding to ERM family members

Ezrin is a member of the Band 4.1 superfamily of cytoskeletal proteins which also includes talin, merlin, radixin and moesin (Gould *et al.*, 1989; Turunen *et al.*, 1989; Lankes and Furthmayer, 1991; Sato *et al.*, 1992). All of the Band 4.1 proteins contain significant homologies in their amino-terminal regions, which have been implicated in membrane association (Arpin *et al.*, 1994). Ezrin, radixin and moesin represent a subfamily of proteins (the ERM family) possessing a higher degree of homology across their entire sequences (Sato *et al.*, 1992). We investigated the capacity of recombinant human ezrin, radixin and moesin produced in a Sf9 baculovirus expression system to bind $[^{32}P]R_{II}$ (Figure 2). All three of the recombinant ERM proteins bound R_{II}. Previous studies have shown that synthetic peptides containing amphipathic α-helical R_{II} binding motifs can block $[^{32}P]R_{II}$ binding to AKAPs (Carr *et al.*, 1991, 1992a). One such peptide containing the R_{II} binding region of the thyroid AKAP, HT-31, binds with especially high affinity (Carr *et al.*, 1991, 1992a) and has been used to identify authentic R_{II} binding proteins. We have reported previously that the HT-31 peptide inhibited R_{II} binding to AKAP78 (Dransfield *et al.*, 1995). A 1 μM concentration of the HT-31 peptide inhibited binding of $[^{32}P]R_{II}$ to all three ERM proteins (Figure 2).

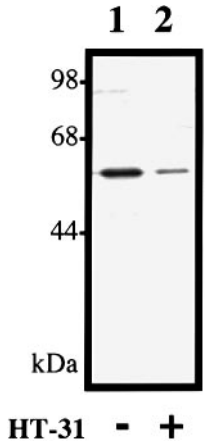

Fig. 3. Binding of [^{32}P]R_{II} to ezrin affinity resin. Ezrin affinity resin was incubated with [^{32}P]R_{II} (1) in the absence or (2) in the presence of 1 μM HT-31 inhibitory peptide. The positions of molecular mass standards (kDa) are noted at the left. The autoradiograph of adherent proteins resolved on 10% SDS–PAGE gels demonstrates the inhibition of [^{32}P]R_{II} binding to ezrin by the HT-31 peptide. The results are representative of two separate experiments.

Binding of R_{II} to ezrin affinity resin

To confirm that the binding of R_{II} to ezrin was not an artifact of the electroblot overlay assay, we incubated recombinant ezrin affinity resin with [^{32}P]R_{II} in the absence or presence of the HT-31 inhibitory peptide. Figure 3 demonstrates that [^{32}P]R_{II} was retained on the ezrin affinity resin. However, the HT-31 peptide significantly inhibited the association of R_{II} with ezrin. These results, along with those in Figure 1, demonstrate that R_{II} and ezrin associate under non-denaturing conditions.

Mapping the R_{II} binding domain in ezrin

All three of the ERM proteins contain an amino-terminal Band 4.1 homology domain (Sato *et al.*, 1992; Algrain *et al.*, 1993; Arpin *et al.*, 1994; Martin *et al.*, 1995). This is followed by a region of α-helix which, in ezrin and radixin, is terminated by a polyproline domain. Following the polyproline domain is a carboxy-terminal domain which has been implicated in actin filament association (Turunen *et al.*, 1994). To map the region responsible for R_{II} binding, we investigated R_{II} binding to full-length recombinant ezrin and to a series of ezrin truncations produced in a Sf9 baculovirus expression system. Figure 3 summarizes the results of binding to these constructs. R_{II} binding was observed in full-length recombinant ezrin and a carboxy-terminal truncation ($ezrin_{310–586}$), but no binding was observed in the amino-terminal fragment ($ezrin_{1–310}$). Similar results were obtained in full-length, amino and carboxyl truncations of ezrin expressed as GST fusion proteins in a prokaryotic expression system (data not shown). Further carboxy-terminal truncations mapped the R_{II} binding to the region of predicted α-helix between amino acids 373 and 478 (Figure 4). These results suggested that the R_{II} binding region was situated within the ezrin α-helical domain. No binding of [^{32}P]R_{II} was demonstrated to the recombinant subunits of the H/K-ATPase (Figure 4), supporting the specificity of the interaction of R_{II} with a discrete component of the parietal cell secretory machinery, ezrin.

To define further the region responsible for R_{II} binding in

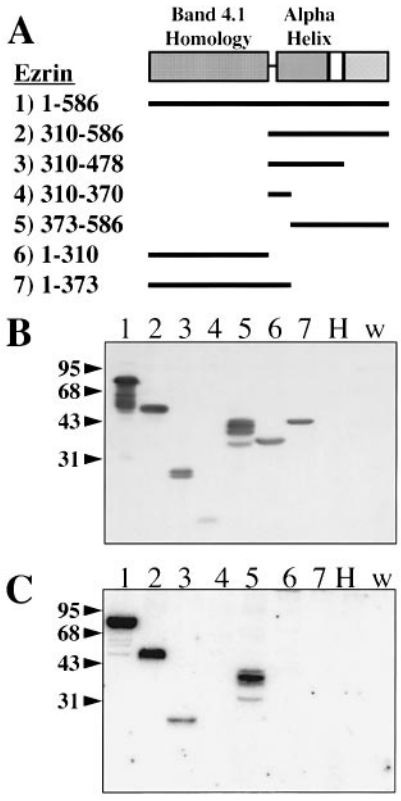

Fig. 4. Binding of [^{32}P]R_{II} to recombinant ezrin constructs. (**A**) Total extracts of 20 000 Sf9 cells expressing full-length recombinant ezrin (A.1) six truncated constructs (indicated as A.2–7), the α and β subunits of H/K-ATPase (H) and wild-type viral proteins (w) were resolved on 15% SDS–PAGE and transferred to nitrocellulose. (**B**) Visualization of ezrin constructs with a combination of polyclonal rabbit anti-ezrin antibodies. The Western blot demonstrates the migration of all seven recombinant protein constructs. (**C**) [^{32}P]R_{II} overlay: the autoradiograph demonstrates R_{II} binding to full-length ezrin, $ezrin_{310–586}$, $ezrin_{310–478}$ and $ezrin_{373–586}$. Minor binding to some proteolytic fragments was also noted. This pattern of binding to the truncated constructs mapped the binding site to between amino acids 373 and 478. No binding was observed either in the Sf9 extract containing the α and β subunits of H/K-ATPase or in the extract from cells infected with wild-type baculovirus. The results are representative of two separate experiments.

ezrin, a series of recombinant carboxy-terminal truncations were constructed in *Escherichia coli* (Figure 5). In both purified GST fusion proteins and cleaved recombinant proteins, R_{II} binding was observed in $ezrin_{1–439}$, $ezrin_{1–466}$ and $ezrin_{1–482}$, but not in the shorter truncations, $ezrin_{1–366}$, $ezrin_{1–406}$ and $ezrin_{1–423}$. No binding of [^{32}P]R_{II} to the GST polypeptide was observed following cleavage of the fusion proteins (Figure 5). These results, in combination with the binding to the Sf9 truncations, map the R_{II} binding region to between amino acids 373 and 439.

Since previous investigations have suggested that R_{II} association with AKAPs is mediated through an amphipathic α-helical domain (Carr *et al.*, 1991), we examined the region between amino acids 373 and 439 for a putative R_{II} binding site. Figure 6A depicts the helical wheel analysis of a putative amphipathic 14-amino-acid sequence between amino acids 417 and 430. While AKAPs in general have little sequence homology across their entire sequences, significant homologies have been noted in their R_{II} binding regions (Scott and McCartney, 1994). A comparison of the region spanning the R_{II} binding motif

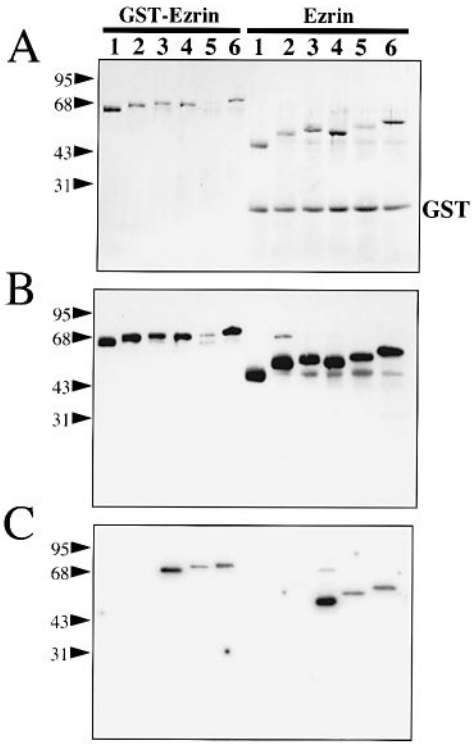

Fig. 5. Binding of $[^{32}P]R_{II}$ to carboxy-terminal truncations of ezrin. Purified recombinant truncations as GST fusion proteins (GST–ezrin) or after cleavage of the GST extension (ezrin) were separated on 8% SDS–PAGE gels (1, $ezrin_{1–366}$; 2, $ezrin_{1–406}$; 3, $ezrin_{1–423}$; 4, $ezrin_{1–439}$; 5, $ezrin_{1–466}$; 6, $ezrin_{1–482}$). (**A**) Coomassie blue-stained gel of proteins. The position of the GST cleavage product is noted at the right. $Ezrin_{1–439}$ repeatedly demonstrated a slightly higher mobility than predicted. (**B**) Ezrin immunoblot. Proteins were transferred to nitrocellulose and probed with a combination of anti-ezrin polyclonal antibodies. Ezrin immunoreactivity coincided with the Coomassie blue staining bands observed in (A). (**C**) Binding of $[^{32}P]R_{II}$. Proteins were transferred to nitrocellulose and probed with $[^{32}P]R_{II}$. Only the three largest ezrin constructs bound R_{II}. The results are representative of two separate experiments.

of ezrin with analogous regions from MAP2, HT-31 and AKAP79 (Figure 6B) shows significant homologies throughout the sequence.

As noted above, peptides constructed against the amphipathic helical regions of AKAPs can inhibit R_{II} binding to native AKAPs. The HT-31 amphipathic helix peptide inhibited R_{II} binding to purified gastric AKAP78/ezrin (Figure 6C). We synthesized a 30-amino-acid polypeptide encompassing the predicted ezrin amphipathic α-helix ($ezrin_{409–438}$). Figure 5C demonstrates that $ezrin_{409–438}$ could block all R_{II} binding to purified gastric ezrin (IC_{50} = 30 μM). While the IC_{50} for $ezrin_{409–438}$ was higher than that for the HT-31 peptide, this figure is similar to inhibitory concentrations for other AKAP R_{II} binding domain peptides (Carr *et al.*, 1992b). In addition, previous studies have also demonstrated that disruption of the amphipathic helical region with leucine to proline substitutions abolishes R_{II} binding. We, therefore, tested the ability of a synthetic $ezrin_{409–438}$ peptide with a leucine to proline substitution at amino acid 421 ($ezrin_{409–438L>P}$) to inhibit R_{II} binding to purified gastric AKAP78/ezrin (Figure 6). While 60 μM $ezrin_{409–438}$ completely inhibited R_{II} binding, $ezrin_{409–438L>P}$ failed to inhibit at concentrations of up to 100 μM. These data support the presence of an amphipathic α-helical R_{II} binding site in a specific region of the ezrin α-helical domain.

Localization of R_{II} in parietal cells

To investigate the localization of R_{II} in parietal cells, we developed a polyclonal rabbit antiserum against recombinant murine R_{II}. In Western blots, the polyclonal antibodies recognized a single 55 kDa species in murine fundic mucosa which co-migrated with recombinant murine R_{II} (Figure 7).

The polyclonal antibodies were used to study the localization of R_{II} in parietal cells from vehicle-treated and gastrin-stimulated mice. Strong staining for R_{II} was observed in mouse parietal cells in a punctate pattern throughout the cell (Figure 8a). No staining of parietal cells was observed with pre-immune serum (data not shown). Double labeling of F-actin with bodipy-phallacidin demonstrated scattered areas of overlap. Only occasional parietal cells in vehicle animals also showed co-localization of R_{II}, and these were found predominantly near the tops of glands (data not shown). In gastrin-stimulated animals, many parietal cells exhibited co-localization of R_{II} and bodipy-phallacidin staining (Figure 8d–i). No labeling was observed in the nuclei of parietal cells from either vehicle or gastrin-treated animals. These data suggested an association of R_{II} with the secretory canaliculus, at least in stimulated parietal cells.

Discussion

In many cellular systems, type II A-kinase is sequestered in specific regions of the cell. This compartmentalization may regulate the spatial and temporal access of the kinase to specific substrates as well as to its activator, cAMP. Investigations over the past several years have pointed to the presence of a diverse group of AKAPs which are responsible for immobilizing type II A-kinase in discrete particulate fractions in association with either membrane organelles or the cytoskeleton (Scott *et al.*, 1992). In gastric parietal cells, histamine, through the elevation of intracellular cAMP, stimulates the fusion of intracellular tubulovesicles containing H/K-ATPase with an apically oriented intracellular canalicular target membrane system (Forte *et al.*, 1981). In parietal cells, we previously have provided evidence that elevations in cAMP lead to dephosphorylation of R_{II} in particulate fractions (Goldenring *et al.*, 1992). These studies had suggested an association of type II A-kinase with the intracellular canalicular membranes. In addition, our preliminary investigations demonstrated an association of the major parietal cell AKAP, AKAP78, with the Triton X-100 cytoskeleton (Dransfield *et al.*, 1995). The present results indicate that R_{II} relocates to the secretory canaliculus during stimulation of parietal cells. These data provided further support for the association of the type II A-kinase with the canalicular membrane, which is intimately associated with a dense F-actin cytoskeleton (Hanzel *et al.*, 1989; Soroka *et al.*, 1993). The present studies demonstrate that AKAP78 is ezrin, the actin-associated protein which is highly enriched in the F-actin cytoskeleton underlying the parietal cell canaliculus. Thus, ezrin may anchor the type II A-kinase into the critical zone for regulated

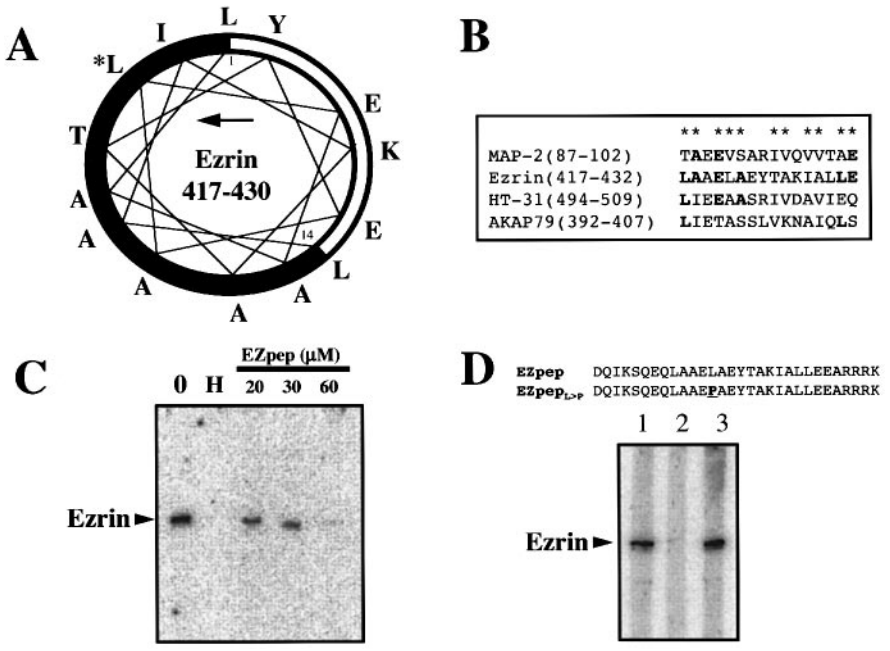

Fig. 6. Analysis of the putative R_{II} binding, amphipathic α-helical region of ezrin. (**A**) Helical wheel analysis of the ezrin amino acid sequence between 373 and 478 demonstrated a single 14-amino-acid region which displayed a putative amphipathic α-helical structure. *L indicates the position of the leucine to proline substitution which disrupts the amphipathic helix. (**B**) The putative R_{II} binding region of ezrin was compared with analogous regions from MAP-2, HT-31 and AKAP79. Positions of significant ezrin homology with other AKAP sequences are designated with asterisks (*). Positions of identities with the ezrin sequence are noted in bold. (**C**) To analyze the amphipathic α-helical structure, purified gastric AKAP78/ezrin was resolved on 8% SDS–PAGE gels and transferred to nitrocellulose. Strips of electroblot (~0.5 μg of AKAP78/ezrin protein) were incubated with $[^{32}P]R_{II}$ in the absence of blocking peptide (0) or in the presence of 1 μM HT-31 peptide or $ezrin_{409–438}$ peptide (EZpep) in concentrations of 20, 30 and 60 μM. The HT-31 peptide abolished binding to AKAP78. $Ezrin_{409–438}$ peptide blocked binding to ezrin with an approximate IC_{50} of 30 μM. The results are representative of four separate experiments. (**D**) Disruption of the α-helical domain with a leucine to proline substitution. A synthetic peptide with a leucine to proline substitution at amino acid 421 in $ezrin_{409–438}$ ($ezrin_{409–438L>P}$) was constructed. Strips of electroblot (~0.5 μg of AKAP78/ezrin protein) were incubated with $[^{32}P]R_{II}$ in the absence of blocking peptide (1), in the presence of 60 μM $ezrin_{409–438}$ (2) or in the presence of 60 μM $ezrin_{409–438L>P}$ (3). $Ezrin_{409–438L>P}$ failed to inhibit binding to AKAP78/ezrin. The results are representative of three separate experiments.

fusion of intracellular vesicles with the canalicular target membranes.

A great deal of study has focused on the functions of the amino and carboxyl domains of ezrin. The Band 4.1 homology region has been implicated in the association of ezrin with membrane surfaces. In particular, recent investigations have suggested that, in some systems, the ezrin interacts with the ubiquitous cell surface glycoprotein, CD44 (Tsukita *et al.*, 1994). The carboxy-terminal region of ezrin has been implicated in the association of ezrin with F-actin filaments. Ezrin may interact preferentially with F-actin filaments containing b-actin (Yao *et al.*, 1995). While co-localization of ezrin with actin filaments has been noted in a number of systems (Hanzel *et al.*, 1991; Algrain *et al.*, 1993; Arpin *et al.*, 1994; Martin *et al.*, 1995), the exact nature of the ezrin–F-actin interaction is controversial. Turunen *et al.* (1994) have supported a direct association of ezrin with F-actin through its terminal 35 amino acids, while other studies (Shuster and Herman, 1995) suggest an indirect association with F-actin. Several investigations also suggest that the amino- and carboxy-terminal regions interact to regulate their respective activities. Martin *et al.* (1995) have demonstrated that the ezrin amino-terminal domain inhibits the capacity of the carboxy-terminus to induce cellular extensions. This inhibition may, in part, stem from a capacity of the protein to form head-to-tail homodimers (Gary and Bretscher, 1993, 1995; Andreoli *et al.*, 1994).

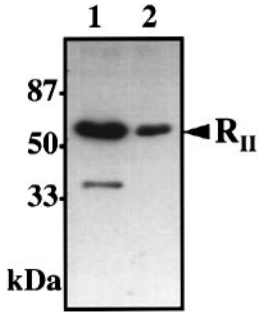

Fig. 7. Characterization of polyclonal anti-murine R_{II}. The rabbit polyclonal antiserum raised against recombinant murine R_{II} (1:6000) was used to probe Western blots of (1) recombinant murine R_{II} (50 ng) and (2) post-nuclear supernate from homogenates of murine fundic mucosa (100 μg). Specific labeling was detected with horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescence. The positions of molecular mass standards (kDa) are noted at the left. A single 55 kDa immunoreactive band was observed in mouse fundic mucosa which co-migrated with recombinant murine R_{II}. The results are representative of two separate experiments.

The present studies suggest that the α-helical region of ezrin contains a domain for association with type II A-kinase. Both recombinant radixin and moesin also bound R_{II}. Since the R_{II} binding to all three ERM proteins was inhibited by the HT-31 amphipathic helix peptide, anchoring of A-kinase may be a general property of the

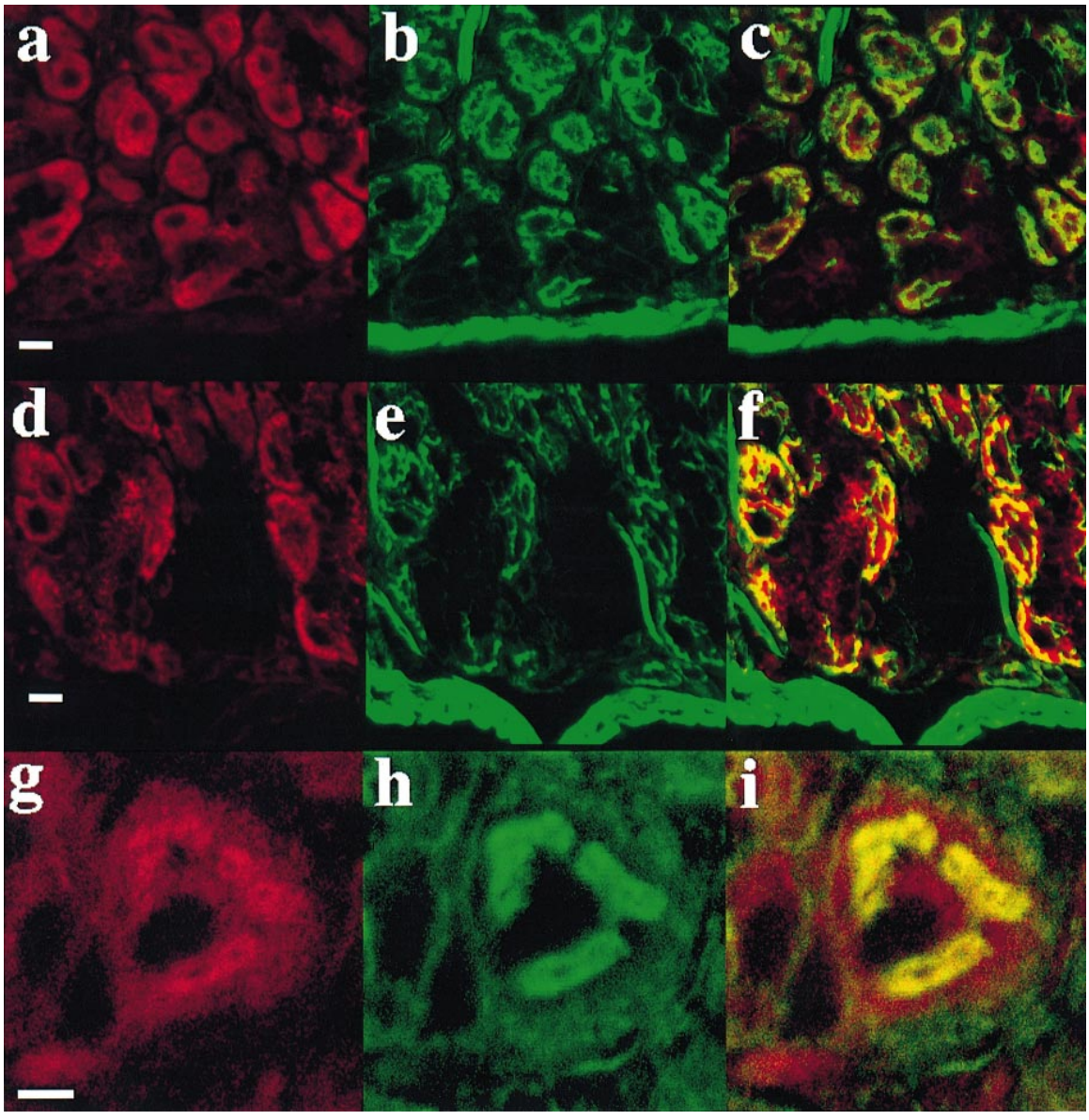

Fig. 8. Localization of R_{II} in murine gastric parietal cells. Polyclonal antibodies against R_{II} (1:6000) were used to stain sections of murine fundic mucosa from vehicle-treated mice (**a**–**c**) and mice stimulated with gastrin (**d**–**i**). R_{II} staining was detected with Cy5-rabbit IgG (a, d and g). All sections were double labeled with bodipy-phallacidin (b, e and h) to detect F-actin which underlies the secretory canaliculus. Anaglyph superimpositions of the double-staining patterns are shown in (c), (f) and (i). R_{II} co-localization with F-actin lining the secretory canaliculus is observed prominently in parietal cells from animals stimulated with gastrin (f and i). The results are representative of four separate experiments. Bar = 20 μm.

ERM proteins. Our results have delineated a specific putative amphipathic α-helical region in ezrin between amino acids 417 and 430. The requirement for the α-helix in binding is confirmed by the inability of the proline-substituted ezrin peptide ($ezrin_{409–438L>P}$) to block R_{II} binding to ezrin. Since the amphipathic α-helix peptide from HT-31 associates with the amino-terminus of R_{II} (Carr *et al.*, 1991; Carr and Scott, 1992), its ability to block R_{II} binding to ezrin indicates that ezrin also binds to the same AKAP association domain. The specificity and structural basis for the interaction of ezrin with R_{II} was established by the analysis of recombinant truncations and binding inhibition with synthetic peptides spanning the putative amphipathic α-helix. Although these studies indicate that ezrin is the major AKAP in parietal cells, the elution of cytosolic ezrin from R_{II} affinity resin with 250 mM NaCl suggests that it has a lower affinity than many previously described AKAPs (Scott and McCartney, 1994). While these studies focused on soluble ezrin isolated following homogenization, *in situ* most of the ezrin in parietal cells is associated with the F-actin cytoskeleton (Soroka *et al.*, 1993). Mandel and colleagues (Chen *et al.*, 1994) have observed that dephosphorylation of ezrin during renal hypoxia correlates with dissociation of ezrin from cortical actin filaments. Post-translational modifications in cytoskeletally associated ezrin might alter affinities for R_{II}. Since terminal truncations of ezrin can bind R_{II}, dimerization of ezrin is not required. Nevertheless, it is not clear whether dimerization could inhibit binding or whether binding of R_{II} could inhibit dimeriz-

ation. Association with non-dimerized or phosphorylated ezrin might result in altered affinities for the interaction of R_{II} with ezrin.

In addition to its established role as a membrane–actin bridging protein, several studies have suggested important interactions between ezrin and second messenger systems. Bretscher (1989) demonstrated that epidermal growth factor (EGF) stimulated the phosphorylation of both tyrosine and serine residues on ezrin, in concert with its stimulated recruitment into zones of membrane ruffling in A-431 cells. Histamine elicits the phosphorylation of ezrin *in situ* in parietal cells on serine residues (Urushidani *et al.*, 1989). In addition, EGF stimulates ezrin phosphorylation in parietal cells on a serine residue distinct from that phosphorylated in response to histamine (Whitney *et al.*, 1990). While tyrosine phosphorylation of ezrin in A-431 cells appears to be due to phosphorylation by the EGF receptor tyrosine kinase (Bretscher, 1989), the kinases responsible for serine phosphorylation are as yet unclear. In addition to its status as a substrate for protein kinases, recent investigations have demonstrated that a PIP_2 binding site is contained within the first 309 amino acids of ezrin (Niggli *et al.*, 1995). While the status of ezrin as a target for protein phosphorylation is well established, the present data represent the first indication that ezrin, as well as other ERM proteins, directly associates with a specific protein kinase. Thus, ezrin may serve to sequester type II cAMP-dependent protein kinase within critical sub-apical membrane domains providing for specific localization of A-kinase action within epithelial cells. Such regionalization in parietal cells to the intracellular canaliculus would position A-kinase in the zone where membrane fusion and recycling are thought to be regulated by elevations of intracellular cAMP. Although widely accepted, the membrane fusion and recycling hypothesis (Forte *et al.*, 1981) is still in dispute and has been challenged recently by an alternative mechanism involving an expansion of the secretory membrane (Pettitt *et al.*, 1995). Nevertheless, in both mechanisms proposed, ezrin phosphorylation is considered a key factor for membrane reorganization during parietal cell stimulation. In other epithelial cells, sequestration of A-kinase by ezrin might provide for specific regulation of plasma membrane pumps and channels located in the proximity of sub-plasmalemmal F-actin. Thus, ezrin may not only link plasma membrane with the cytoskeleton, but also act as a nidus for the localization of critical regulatory enzyme systems.

Materials and methods

Materials

[γ-^{32}P]ATP was purchased from New England Nuclear (Boston, MA). New Zealand White rabbits (2–3 kg) were purchased from Shelton's Bunny Barn (Waverly Hall, GA). A-kinase catalytic subunit was purchased from Promega (Madison, WI). All other reagents were from standard suppliers and were of the highest purity available.

Gastric mucosal fractionation

Scraped fundic gastric mucosa from the rabbit was minced and diluted 10-fold (v/v) with homogenization buffer (buffer A) in (mM): mannitol (113), sucrose (37), EDTA (0.4), MES (5), pH 6.7, containing benzamidine (5), AEBSF (0.1), 0.5 mg/ml leupeptin, 0.2 mg/ml chymostatin, 0.2 mg/ml pepstatin, 0.5 mg/ml soybean trypsin inhibitor and 1.4 TIU aprotinin. The tissue samples were homogenized in a Potter Teflon-on-glass homogenizer by five up and down strokes at 300 r.p.m. with a Cole Parmer Master Servodyne homogenizer and sequentially centrifuged at 50, 1000, 10 000 and 100 000 *g* to prepare four particulate pellets and a final high-speed supernate fraction.

Purification of gastric AKAP78

Recombinant R_{II} produced in BL21(DE3)pLysS bacteria possessing an R_{II}-pET11d expression plasmid (a gift of Dr John Scott, Vollum Institute, Portland, OR) was purified by cAMP affinity chromatography. For R_{II} affinity chromatography, R_{II} was not eluted from 0.5 ml of cAMP resin and, after extensive washing in buffer containing 1 M NaCl, the column was equilibrated in 50 mM HEPES, pH 7.4 with the previously noted protease inhibitors (Equilibration buffer). Gastric supernates from two rabbit stomachs were passed over the column and, following extensive washing with Equilibration buffer with monitoring of A_{280}, the column was washed sequentially with Equilibration buffer containing 250 mM, 500 mM and 1 M NaCl, followed by an overnight incubation in the presence of 10 mM cAMP. Fractions of 1 ml were collected.

R_{II} overlay

^{32}P-Labeled R_{II} was used for the overlay procedure. Recombinant R_{II} was phosphorylated by incubation with purified bovine A-kinase catalytic subunit and [γ-^{32}P]ATP, as described previously (Dransfield *et al.*, 1995). Gastric proteins were resolved on 8% SDS–PAGE gels and transferred to nitrocellulose (0.2 μm) overnight at 100 mA. Protein electroblots were blocked with 5% non-fat dry milk, 0.1% bovine serum albumin (BSA) in 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% NaN_3 (Blotto) for 16–24 h at 4°C prior to the addition of [^{32}P]R_{II} (100 000 c.p.m./ml Blotto, supplemented with 5 mM benzamidine) for 4 h at room temperature. Following five 15 min washes in Blotto, binding was visualized by exposure of blots to a Phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA).

Recombinant protein constructs

Recombinant baculoviruses coding for human ezrin, ezrin truncations and rat H/K-ATPase were utilized as previously described (Martin and Mangeat, 1994; Martin *et al.*, 1995). New ezrin constructs ($ezrin_{310–478}$, $ezrin_{1–310}$ and $ezrin_{1–373}$) and viruses coding for human radixin (carboxy-tagged with 11 amino acids of the vesicular stomatitis virus glycoprotein G) and moesin (with amino acids 1–11 deleted) were obtained using similar protocols (Martin and Mangeat, 1994; Martin *et al.*, 1995). Total extracts from Sf9 cells expressing the various recombinant proteins were resolved on SDS–PAGE and transferred to nitrocellulose for 100 min at 0.8 mA/cm^2 in a semi-dry apparatus.

Carboxy-terminal truncations of ezrin were prepared as prokaryotic recombinant proteins. The subcloning of full-length human ezrin cDNA into pGEX-2T vector was described previously (Andreoli *et al.*, 1994). Carboxy-terminal deletions were obtained using the double-stranded nested deletion kit from Pharmacia. The ezrin-containing pGEX-2T vector was cleaved with *Eco*RI, blunted with thio-dNTPs and cleaved with *Esp*I. Exonuclease III was then added and the reaction was stopped every 30 s for 10 min. After nuclease S1 action, filling-in with the Klenow DNA polymerase fragment and overnight ligation, DNA was transformed into TG1 bacteria. Randomly picked clones were grown overnight in LB medium, induced for 1 h with 0.5 mM IPTG and analyzed by Western blotting for protein expression after adsorption of the bacterial lysate to glutathione–agarose. Antibodies against the ezrin moiety or the GST part of the fusion protein were used to select clones according to their relative mobility in SDS–PAGE. Selected clones were sequenced using the T7 Sequenase version 2.0 (Amersham). The full-length ezrin used its own stop codon. The pGEX-2T vector which contains stop codons in all three reading frames provided the stop codon for the truncated proteins. As a consequence, 2–7 additional amino acids encoded by the vector were added after the carboxy-terminal deletion site.

Association of R_{II} with ezrin under non-denaturing conditions

GST–ezrin was purified from *E.coli* as described by Andreoli *et al.* (1994). After extensive dialysis against 100 mM $NaHCO_3$, 15 mg of fusion protein was coupled overnight at 4°C to 6 ml of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's recommendations. After blocking of free sites and washing, the resin was stored in phosphate-buffered saline (PBS) with 0.05% sodium azide. Ezrin affinity beads were blocked with 1 mg/ml BSA in PBS (BSA–PBS) for 1 h at room temperature. Thirty μl of affinity resin was incubated with 250 000 c.p.m. of [^{32}P]R_{II} in 1 ml of BSA–PBS for 1 h at room temperature with constant gentle agitation. Incubations were performed in the presence or absence of 1 μM HT-31 peptide. Following

the incubation, the beads were washed four times in 0.5 ml of PBS and adherent proteins were removed with boiling 1.5% SDS stop solution. Proteins were resolved on 10% SDS–PAGE gels, and dried gels were exposed to Phosphorimaging screens (Molecular Dynamics, Sunnyvale, CA) for 1 h.

Immunoblotting

For the detection of ezrin, proteins were resolved on 8% SDS–PAGE gels and transferred to Immobilon P (Millipore, Bedford, MA). The blots were blocked for 1 h at room temperature with 5% non-fat dry milk in 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% NaN_3 (5% Blotto). The blots were then probed in 3% non-fat dry milk in 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% NaN_3, 0.05% Tween-20 (3% Blotto) for 2 h at room temperature with a monoclonal antibody against ezrin (4C4; 1:2000; a gift from Dr John Forte, University of California, Berkeley). The antibody is specific for ezrin, and does not detect either radixin or moesin. For detection of recombinant ezrin protein constructs, blots were probed with a mixture of polyclonal anti-ezrin antibodies (predominantly anti-carboxy-terminal domain) and anti-ezrin$_{1–310}$, as previously described (Andreoli *et al.*, 1994). After the primary incubation, the blots were washed three times for 15 min each with 3% Blotto and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000) for 1 h at room temperature. The blots were finally washed three times for 15 min each with 3% Blotto and immunoreactivity was detected with chemiluminescence (Renaissance, NEN) and autoradiography.

AKAP peptide competition

HT-31 peptide, ezrin$_{409–438}$ peptide (DQIKSQEQLAAELAEYTAKIALLEEARRRK) and ezrin$_{409–438L>P}$ (DQIKSQEQLAAEPAEYTAKIALLEEARRRK) were synthesized as free acids by the University of Georgia, Athens Peptide Synthesis Core Laboratory. Peptides were dissolved (1 mM) in distilled water. For peptide competition experiments, [^{32}P]R_{II} was pre-incubated for 30 min either in the absence or presence of different concentrations of competitive peptide, prior to its addition to strips of electroblots of purified gastric ezrin.

R_{II} antibodies

A polyclonal antiserum against recombinant murine R_{II} was developed in New Zealand White rabbits. To verify the specificity of the antiserum, Western blots of recombinant R_{II} and homogenates of mouse gastric mucosa were probed at a 1:6000 dilution. Specific binding was detected with anti-rabbit horseradish peroxidase secondary antibodies and enhanced chemiluminescent detection.

R_{II} immunocytochemistry

C57/Bl6 mice (Jackson Laboratories) were injected intraperitoneally with either saline vehicle or gastrin (50 μg/kg). After 30 min, animals were sacrificed by carbon dioxide inhalation, and the stomach was removed rapidly, incised along the greater curvature and fixed in 4% paraformaldehyde. Frozen sections of stomachs were cut at 5 μm thickness and stained with either pre-immune serum or the anti-R_{II} antiserum (1:6000) for 2 h at room temperature. Specific labeling was visualized with Cy5-conjugated donkey anti-rabbit IgG (Jackson Laboratories). Sections were also labeled with bodipy-phallacidin (Molecular Probes) to visualize F-actin underlying the secretory canaliculus. Sections were examined on a Molecular Dynamics confocal fluorescence microscope with dual imaging using Imagespace software.

Acknowledgements

We thank Dr John Scott for the gift of recombinant murine R_{II}, Dr John Forte for the gift of a monoclonal antibody against ezrin, Dr Monique Arpin for the human radixin cDNA, and Dr Gilles Thomas for the human moesin cDNA. The authors thank Dr Richard Cameron for assistance with the production of polyclonal antibodies against R_{II}. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) FIRST Award Grant DK43405, and a Veterans Affairs Merit Award (to J.R.G.). D.T.D. was the recipient of a National Research Service Award DK-09026. P.H.M. is supported by a grant from l'Association pour la Recherche sur le Cancer (Contract 6844) and grant BMH4 CT950090 from the European Commission.

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Received on June 14, 1996; revised on September 25, 1996