11. S. K. Dower *et al.*, *J. Immunol.* **142**, 4314 (1989).
12. B. M. Curtis *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3045 (1989).
13. D. K. Trager *et al.*, *Transplantation* **47**, 587 (1989).
14. J. W. Kupiec-Weglinski, T. Diamantstein, N. L. Tilney, T. B. Strom, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2624 (1986).
15. R. L. Kirkman *et al.*, *J. Exp. Med.* **162**, 358 (1985).
16. S. C. Grebe and J. W. Streilein, *Adv. Immunol.* **22**, 119 (1976).
17. R. H. Didlake, E. K. Kim, K. Sheehan, R. D. Schreiber, B. D. Kahan, *Transplantation* **45**, 222 (1988).
18. W. C. Fanslow, unpublished data.
19. R. I. Fulmer, A. T. Cramer, R. A. Liebelt, A. G. Liebelt, *Am. J. Anat.* **113**, 273 (1963).
20. C. T. Van Buren, A. Kulkarni, F. Rudolph, *Transplant. Proc.* **15**, 2967 (1983).
21. J.-C. Cerrottini, H. D. Engers, H. R. MacDonald, K. T. Brunner, *J. Exp. Med.* **140**, 703 (1974).
22. V. W. Twist and R. D. Barnes, *Transplantation* **15**, 182 (1973).
23. The indicated dose of sIL-1R was administered together with 100 ng of MSA by intraperitoneal injection on days −1 and 0 and by subcutaneous injection on days 1 and 2, relative to antigen administration, except where otherwise noted. Control mice received MSA but no IL-1R. Specific pathogen–free 8- to 12-week-old BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were obtained from the Jackson Laboratory. MSA was obtained from Sigma and used at a concentration of 1 μg/ml in pyrogen-free saline. Recombinant human IL-1α was generated in *Escherichia coli* and purified to homogeneity as described [S. R. Kronheim *et al.*, *Bio/Technology* **4**, 1078 (1986)]. Recombinant soluble IL-1R was expressed in HeLa cells and purified as outlined (*11*). IL-1α and sIL-1R were diluted in MSA to the appropriate concentration before injection. These diluted cytokine–cytokine receptor preparations always resulted in less than 20 pg of lipopolysaccharide endotoxin being delivered per treatment as measured by the *Limulus* amoebocyte assay (M.A. Bioproducts).
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Phosphorylation of the Polymeric Immunoglobulin Receptor Required for Its Efficient Transcytosis

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The endosomal compartment of polarized epithelial cells is a major crossroads for membrane traffic. Proteins entering this compartment from the cell surface are sorted for transport to one of several destinations: recycling to the original cell surface, targeting to lysosomes for degradation, or transcytosis to the opposite surface. The polymeric immunoglobulin receptor (pIgR), which is normally transcytosed from the basolateral to the apical surface, was used as a model to dissect the signals that mediate this sorting event. When exogenous receptor was expressed in Madin-Darby Canine Kidney (MDCK) cells, it was shown that phosphorylation of pIgR at the serine residue at position 664 is required for efficient transcytosis. Replacement of this serine with alanine generated a receptor that is transcytosed only slowly, and appears to be recycled. Conversely, substitution with aspartic acid (which mimics the negative charge of the phosphate group) results in rapid transcytosis. It was concluded that phosphorylation is the signal that directs the pIgR from the endosome into the transcytotic pathway.

EPITHELIAL CELLS HAVE TWO SEPARATE plasma membrane domains, apical and basolateral, that are characterized by distinct protein compositions. In MDCK epithelial cells, approximately one-half of the cell surface is internalized per hour (*1, 2*), and sorting of proteins from the endosomal compartment to their appropriate cellular destinations is critical for the maintenance of cell polarity. From this compartment, most membrane proteins are either recycled to the original cell surface (for example, transferrin receptor) (*3*) or transported to lysosomes for degradation [for example, epidermal growth factor (EGF) receptor] (*4*). Certain proteins, however, are segregated into separate vesicles and transcytosed to the opposite cell surface, presumably because they contain sorting signals that direct them into this transcytotic pathway. The pIgR, which transports immunoglobulin A (IgA) and immunoglobulin M (IgM) across a variety of epithelia, is a useful model for the study of signals that determine transcytosis (*5, 6*). Newly synthesized pIgR is delivered from the trans Golgi network (TGN) to the basolateral cell surface where it can bind ligand. Receptor-ligand complexes (or receptor alone) are rapidly endocytosed and enter basolateral endosomes, where segregation into transcytotic vesicles takes place (*7*). These vesicles are then transported to the apical cell surface, where the extracellular domain of the receptor is proteolytically cleaved and released into secretions. This released receptor fragment is referred to as secretory component (SC).

The nature of the sorting signals that direct pIgR into the transcytotic pathway is not known. In rat liver, the pIgR has been shown to be phosphorylated on serine (*8*), and it is thought that this modification may be important in receptor function. To address this issue directly, we used MDCK cells that stably express cDNA encoding the rabbit pIgR (*9*). The exogenously expressed receptor functions in these cells as in vivo, transporting IgA from the basolateral to the apical surface (*9*). If receptor phosphorylation were important in mediating sorting, it should occur in these cells as well. Immunoprecipitation of pIgR from [^{32}P]P_i (inorganic phosphate)-labeled cells revealed this to be the case (Fig. 1, lane 1). Phosphopeptide mapping (Fig. 1, lane 2) localized most of the incorporated ^{32}P to a peptide containing 4 of the 11 cytoplasmic serines in the rabbit receptor (*10*). We then used site-directed mutagenesis to define the most membrane-proximal of these, Ser^{664}, as the primary phosphorylation site. Mutation of this serine to alanine, a nonphosphorylatable residue, resulted in an 84% reduction in ^{32}P incorporation into the mutant Ala^{664} receptor (*11*) (Fig. 1, lane 3) and a total loss of incorporation into the major phosphopeptide produced after treatment with V8 protease (lane 4). This site is not analogous to those defined for any of the known serine-threonine kinases (*12*).

To analyze the role of phosphorylation at this site in receptor sorting, we made an additional mutation, substituting aspartic acid for the target serine (Asp^{664} receptor). The negatively charged aspartate has been shown to mimic the effect of serine phosphorylation in regulation of the enzyme isocitrate dehydrogenase (*13*), suggesting that it is the charge of the phosphate group that mediates this process. To examine the effects of these mutations on receptor sorting, we assayed the efficiency of sorting of wild-type, Ala^{664}, and Asp^{664} receptors at each of three points in the receptor pathway: vectorial delivery from the TGN to the basolateral cell surface, endocytosis from the cell surface, and postendocytotic sorting from the endosome to other cellular destinations (*14*).

To analyze receptor delivery to the basolateral cell surface, we used a modification of the trypsin sensitivity assay devised by Mat-

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lin and Simons (*15*). Cells expressing either wild-type, Ala^{664}, or Asp^{664} pIgR were pulse-labeled with [^{35}S]cysteine and then incubated in the absence of isotope for 45 min without trypsin (Fig. 2, lanes 1, 3, and 5) or with trypsin (25 μg/ml) (lanes 2, 4, and 6) in the basolateral medium. All three receptor forms are almost completely sensitive to basolateral trypsin over this time period, indicative of vectorial delivery to the basolateral surface. In contrast, a mutant receptor that lacks the entire cytoplasmic domain and is not delivered basolaterally (*16*) is insensitive to basolateral trypsin (Fig. 2, lane 8), demonstrating that under these conditions, receptors that do not reach the basolateral surface are not accessible to protease. These data suggest that delivery of pIgR to the basolateral cell surface is not dependent on receptor phosphorylation.

We next assayed the rate of internalization of receptors from the basolateral cell surface by measuring the rate of a single round of ligand uptake. At 4°C the native ligand, dimeric IgA, produces unacceptably high levels of background binding to the filters on which the cells are cultured; therefore, we used a substitute ligand, ^{125}I-labeled Fab fragments derived from a polyclonal antibody raised against rabbit SC (Fab). The validation of this Fab as a legitimate substitute ligand has been described (*17*). Endocytosis of wild-type, Ala^{664}, and Asp^{664} receptors was uniformly rapid, with a half-time of internalization of <1 min at 37°C (Fig. 3). On the basis of data from both experiments, we conclude that the wild-type and mutant receptors are all delivered efficiently to the basolateral surface and then endocytosed with identical kinetics.

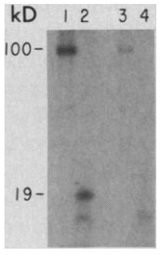

Fig. 1. Identification and mutagenesis of the major phosphorylation site of pIgR. MDCK cells expressing wild-type pIgR were labeled with [^{32}P]P_i (*29*), and pIgR was isolated by immunoprecipitation as previously described (*9*). Immunoprecipitates were divided and one-half of each sample was prepared for electrophoresis without further treatment (lane 1). The other half was digested to completion with staphylococcal V8 protease before electrophoresis (lane 2) under conditions where cleavage is Glu-specific (*30*). The major phosphopeptide (apparent molecular mass of 19 kD) comigrated with the single [^{35}S]methionine-labeled V8 peptide (Asp^{607} to Glu^{678}), which contains the only two methionine residues in the pIgR sequence (18) (*10*). This peptide, which includes the transmembrane domain, contains cytoplasmic serines at positions 664, 667, 673, and 675. To identify the phosphorylated serine, we used oligonucleotide-directed mutagenesis (*31*) to change individual serines to alanine. Complementary DNAs encoding mutant receptors were then expressed in MDCK cells as described (*9*). Cells expressing amounts of mutant receptor similar to that of the wild-type receptor were labeled with [^{32}P]P_i and immunoprecipitated as previously described. One mutant (Ala^{664}) exhibited a marked reduction in phosphate incorporation compared to the wild-type receptor (lane 3). In addition, the 19-kD peptide produced by V8 protease treatments, which contains most of the ^{32}P in wild-type receptor (lane 2), contains no detectable phosphate in the mutant (lane 4). By comparison, mutation of Ser^{667} to Ala causes only a slight reduction in ^{32}P incorporation (*18*) and has no effect on receptor phenotype in any of the assays described here.

The final step in the pathway is the transcytosis of the receptor from the basolateral endosome to the apical cell surface, followed by rapid cleavage and release of SC into the apical medium. To measure this process, we cultured MDCK cells expressing either wild-type, Ala^{664}, or Asp^{664} receptors on permeable filter supports, metabolically labeled them with [^{35}S]cysteine, and then incubated them in medium containing unlabeled cysteine for various periods of time. As described previously, the rate of transport of all three receptor forms to the basolateral endosomal compartment is similar. Therefore, differences in the rate of cleavage to SC should directly reflect perturbations in the transcytotic leg of the receptor's pathway.

As described previously (*9*), the wild-type pIgR is synthesized as a 90-kD precursor (Fig. 4A, lane 1), glycosylated to yield a doublet of 100 to 105 kD (lanes 2, 3, and 4), and, after transcytosis, is proteolytically cleaved to SC, which is released into the apical (lanes 5, 7, and 9) but not basolateral (lanes 6, 8, and 10) medium. After 5 hours of incubation with unlabeled cysteine, 81% of the labeled wild-type receptor molecules appeared in the apical medium as SC (Fig. 4E). The Ala^{664} mutant appeared to be synthesized and glycosylated normally; however, release of SC into the apical medium was reduced, with only 18% of the labeled molecules having been cleaved by 5 hours (Fig. 4B, lanes 5, 7, and 9, and Fig. 4E). The half-life of the cellular form was correspondingly prolonged (Fig. 4D). In contrast, the release of SC from cells expressing the Asp^{664} mutant is accelerated with respect to wild-type pIgR (Fig. 4C, lanes 5, 7, and 9, and Fig. 4E), reaching 82% after only 1 hour.

It is possible that the mutant receptors reach the apical surface with normal kinetics, but that the Ala^{664} receptor is more resistant to cleavage to SC by the endogenous protease, whereas the Asp^{664} receptor is cleaved more rapidly. To test this, we performed an experiment analogous to the trypsin experiment described earlier, except that cells were exposed to apical trypsin for up to 2 hours after removal of isotope. No additional decrease in cell-associated receptor was observed under these conditions when compared to untreated controls (*18*). This shows that uncleaved Ala^{664} receptor does not accumulate at the apical surface, and that the measured rates of SC release for each mutant must therefore accurately reflect their rates of delivery to the apical plasma membrane from the basolateral endosome.

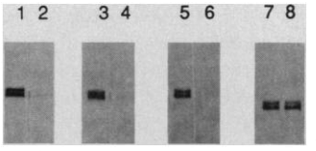

Fig. 2. Determination of biosynthetic targeting to the basolateral cell surface. MDCK cells expressing either wild-type or mutant receptors were cultured on permeable filter supports (Costar Transwells, 0.4 μm, 6.5-mm diameter) as described (*17*), pulse-labeled with [^{35}S]cysteine (3 mCi/ml) for 10 min, and then incubated in MEM–0.6% BSA for 45 min in the absence of label and in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of trypsin (25 μg/ml) (TPCK, Worthington) in the basolateral chamber. Where trypsin was present, soybean trypsin inhibitor (200 μg/ml) was included in the medium bathing the opposite cell surface. Cells were harvested, immunoprecipitated, and processed as previously described (*10*). Immunoprecipitates were analyzed by SDS–polyacylamide gel electrophoresis (SDS-PAGE) (10% gel) and fluorography. Lanes 1 and 2, wild-type pIgR; lanes 3 and 4, Ala^{664} mutant; lanes 5 and 6, Asp^{664} mutant; lanes 7 and 8, a truncated, tail-minus pIgR construct that is delivered directly to the apical surface (*16*).

To confirm these findings independently, we allowed cells to take up ligand (^{125}I-labeled Fab) for 10 min from the basolateral surface and measured the subsequent appearance of ligand in both the apical and basolateral medium. After a 2-hour incubation, cells expressing wild-type receptor had transcytosed 51% of the internalized ligand, whereas 22% was returned to the basolateral medium (Fig. 3, B and C). In contrast, cells expressing the Ala^{664} receptor transcytosed only 20% of the ligand and 34% was recycled. Conversely, 73% of the ligand was transcytosed by cells expressing the Asp^{664} receptor, with only 16% recycling. In all cases, only 3 to 5% of the ligand was converted to trichloroacetic acid (TCA)–soluble material, and the balance of the counts remained associated with the cells after 2 hours.

A possible complication of this experiment is that, as previously reported, Fab (as well as dimeric IgA) dissociates from cell surface receptors quite rapidly at 37°C (*17*). If significant dissociation occurs intracellularly as well, the fate of receptor might be uncoupled from that of ligand. Evidence from several sources argue against this possibility. First, unlike other ligands that dissociate in endosomes (for example, low-densi-

ty lipoprotein) (*19*), very little of the Fab is degraded in lysosomes. Second, the fates of the mutant receptors correlate well with the fate of ligand bound to these receptors. The observed differences in ligand fate are not due to different rates of dissociation from the various receptors, which we have found to be identical at both *p*H 7.4 and 5.3 (*20*). Thus, these data independently support the conclusion that the Ala^{664} receptor is defective in transcytosis, whereas Asp^{664} receptor is transcytosed more efficiently than the wild type. In addition, the data suggest that a subset of internalized receptor (in particular of Ala^{664}) recycles to the basolateral surface.

Finally, we measured the steady-state distribution of the three receptor forms at both apical and basolateral surfaces, by domain-selective biotinylation as described by Lisanti *et al.* (*21*). The ratios of apical to basolateral receptor determined by this method were 0.88 for wild type, 0.10 for Ala^{664}, and 0.97 for Asp^{664} (*18*). These data further support the notion that the Ala^{664} mutant is defective in transcytosis.

In rat liver, the pIgR exists in two post-Golgi forms of 120 kD (phosphorylated) and 116 kD (unphosphorylated) (*8*). Both forms are present at the basolateral (sinusoidal) surface in roughly equal amounts, whereas the 120-kD species appears to be the form that is present in transcytotic vesicles (*22*). Together with the data reported here, these findings suggest that phosphorylation of the pIgR at Ser^{664} acts as a signal for efficient entry into the transcytotic pathway. The addition of the phosphate could alter the interaction of the receptor's cytoplasmic domain with some component of the sorting machinery or could cause a transmembrane conformational change (or oligomerization) that alters interaction with a lumenal "sorting receptor." The fact that aspartic acid can effectively substitute for phosphoserine suggests that it may be the negative charge of the phosphate group that mediates this effect (*13*). It is unlikely that the point mutations described here produce grossly abnormal structures, as their transport to the basolateral surface, ligand binding, and endocytosis are indistinguishable from that of the wild-type receptor. Rather, we suggest that the Ala^{664} receptor closely resembles the unphosphorylated form of the wild-type receptor, and the Asp^{664} receptor resembles the phosphorylated form.

As a model, we propose that phosphorylated pIgR molecules are rapidly segregated from the endosomal compartment into transcytotic vesicles. Unphosphorylated molecules would enter the transcytotic pathway only slowly. These receptors may instead be recycled, but are clearly not shunted into lysosomes. The use of phosphorylation as a molecular switch may allow the cell to regulate transcytosis in response to external stimuli. An example of this might be the methacholine-induced increase in IgA transport that has been observed in the nasal mucosa (*23*). However, the inclusion of saturating concentrations (50 μg/ml) of dimeric IgA in the basolateral medium does not affect either the extent of receptor phosphorylation or the rate of SC release into the apical medium (*18*), indicating that ligand binding alone is not the stimulus.

Many membrane proteins are known to be phosphoproteins, and for some of these

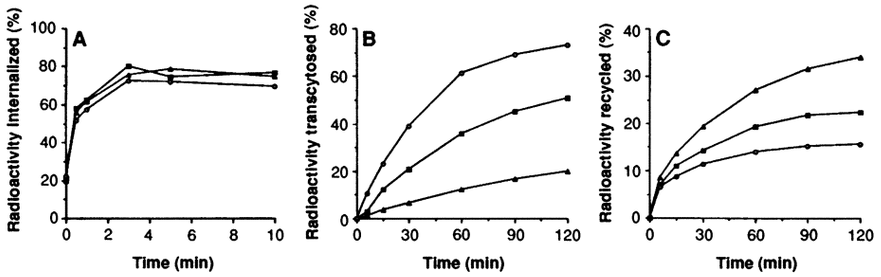

Fig. 3. Internalization and postendocytotic sorting of ligand. (**A**) Determination of endocytic rate. Fab fragments derived from a polyclonal guinea pig antibody to rabbit SC were generated and labeled with ^{125}I as described (*17*). ^{125}I-labeled Fab (4 μg/ml, 4×10^6 cpm/μg, in MEM-BSA) were bound to the basolateral surface of filter-grown MDCK cells expressing either wild-type or mutant pIgR for 2 hours at 4°C, after which the cells were extensively washed with MEM-BSA. The temperature of the filters was brought to 37°C with warmed media for 0 to 10 min, and then they were rapidly cooled to 4°C. The basolateral surface was next exposed to trypsin (50 μg/ml) in MEM-BSA for 60 min at 4°C. After washing, the amount of radioactivity still associated with the cells (intracellular) was determined by removing the filters from their chambers and counting the bound radioactivity directly in a Packard gamma counter. Values are expressed as a percentage of the total amount of radioactivity (cpm) initially bound to the cells before warming. Each point represents the mean of triplicate determinations, which varied by less than 5%. Internalization of Fab by MDCK cells not expressing pIgR was less than 3% that of any of the clones examined. Protease treatment released only 80% of the bound ligand at time zero. (**B** and **C**) Postendocytotic sorting assay. Filter-grown MDCK cells expressing either wild-type or mutant receptors were allowed to bind and internalize ^{125}I-labeled Fab (40 μg/ml, 4×10^6 cpm/μg) from the basolateral surface at 37°C for 10 min. They were then washed quickly four times with warm MEM-BSA and placed into culture wells containing fresh MEM-BSA at 37°C. Apical and basolateral media were removed at various times and replaced with fresh media. Media samples were precipitated with TCA (15% TCA for 1 hour at 0°C) to distinguish Fabs that had been released into the media intact from those that had been degraded intracellularly before release. Filters were cut out and the bound radioactivity was determined directly at the end of the experiment. MDCK cells that do not express pIgR were analyzed in parallel to control for nonspecific uptake of ligand, and the values obtained at each time for these cells (never more than 3.5%) were subtracted as background. Each point represents the average of simultaneous duplicate determinations. Duplicates agreed within 5% and were representative of three separate experiments. Squares, wild-type; triangles, Ala^{664} mutant; and circles, Asp^{664} mutant receptors.

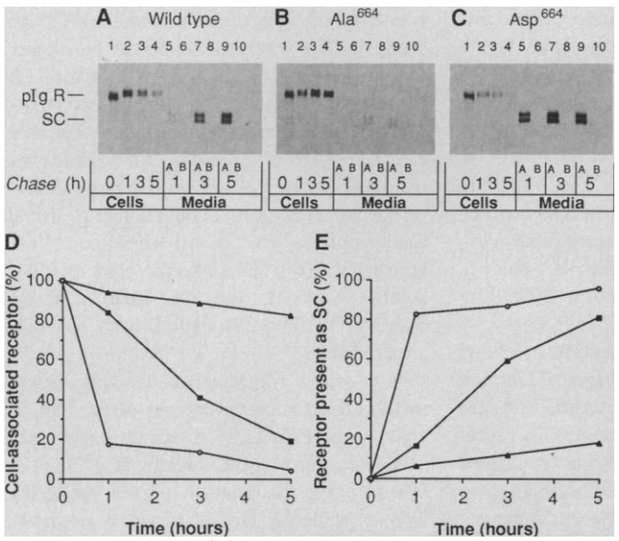

Fig. 4. Pulse-chase analysis of apical delivery of receptor. Filter-grown cells were pulse-labeled as above for 15 min and then incubated in medium without isotope containing 5% FBS for either 1, 3, or 5 hours (h). Cells and media from the apical and basolateral chambers were harvested and immunoprecipitated with antibody to SC. Quantitation of autoradiograms was with an LKB laser densitometer, with multiple exposures in the linear range. For (A) to (C): lanes 1 to 4, immunoprecipitates from cell lysates at 0, 1, 3, and 5 hours of incubation, respectively; lanes 5, 7, and 9 are from the apical media and lanes 6, 8, and 10 are from basolateral media. SC appears as a doublet of about 68 kD in the media. (**A**) Wild-type, (**B**) Ala^{664}, and (**C**) Asp^{664} receptors. (**D**) Quantitation of cell-associated receptor as percentage of total and (**E**) percentage of receptor present as SC. Squares, wild-type; triangles, Ala^{664} mutant; and circles, Asp^{664} mutant receptors.

the role of phosphorylation has been extensively studied (for example, the EGF and insulin receptors). EGF is transcytosed from the basolateral to the apical surface in MDCK cells (*24*) and it is tempting to speculate that phosphorylation of its receptor may have a role in this process. Of course, phosphorylation alone is not sufficient to cause all proteins to be transcytosed. For instance, the tranferrin receptor is phosphorylated, but after endocytosis is recycled exclusively to the basolateral surface of MDCK cells (*3*). Many other membrane proteins are transcytosed from the basolateral to apical surface, including apical proteins in both hepatocytes and enterocytes (*23, 25*). It is not currently known whether such proteins are phosphorylated, although potential phosphorylation sites exist in the cytoplasmic domains of both sucrase isomaltase and dipeptidylpeptidase IV (*26*).

In neonatal rat small intestine, the IgG-Fc receptor transcytoses maternal IgG from the apical to basolateral domain (*27*). Similarly, an isoform of the macrophage-lymphocyte Fc receptor has been shown to undergo transcytosis from the apical to basolateral surface of MDCK cells (*28*). Whether phosphorylation of these receptors is required for entry into the apical to basolateral pathway remains to be determined.

REFERENCES AND NOTES

1. C.-H. von Bonsdorff, S. D. Fuller, K. Simons, *EMBO J.* **4**, 2781 (1985); R. G. Parton, K. Prydz, M. Bomsel, K. Simons, G. Griffiths, *J. Cell Biol.* **109**, 3259 (1989).
2. K. Simons and G. van Meer, *Biochemistry* **27**, 6197 (1988).
3. S. D. Fuller and K. Simons, *J. Cell Biol.* **103**, 1767 (1986).
4. J. Schlessinger *et al.*, *CRC Crit. Rev. Biochem.* **14**, 93 (1983).
5. K. E. Mostov and N. E. Simister, *Cell* **43**, 389 (1985).
6. P. Breitfeld, J. E. Casanova, J. M. Harris, N. E. Simister, K. E. Mostov, *Methods Cell Biol.* **32**, 329 (1989).
7. H. J. Geuze *et al.*, *Cell* **37**, 195 (1984).
8. J. M. Larkin, E. S. Sztul, G. E. Palade, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4759 (1986).
9. K. E. Mostov and D. L. Deitcher, *Cell* **46**, 613 (1986).
10. K. E. Mostov, M. Friedlander, G. Blobel, *Nature* **308**, 37 (1984).
11. Incorporation of ^{32}P into pIgR was quantified, after normalization to [^{35}S]cysteine-labeled immunoprecipitates from parallel plates of cells, by laser densitometry of autoradiograms.
12. A. M. Edelman, D. K. Blumenthal, E. G. Krebs, *Annu. Rev. Biochem.* **56**, 567 (1987).
13. P. E. Thorsness and D. E. Koshland, Jr., *J. Biol. Chem.* **262**, 10422 (1987).
14. The polarity of the clonal cell lines used in these experiments was verified by three independent criteria. (i) Transepithelial resistance (65 ± 21 ohms/cm^2 for wild type, 72 ± 26 ohms/cm^2 for Ala^{664}, and 81 ± 30 ohms/cm^2 for Asp^{664}); the data represent the mean value (± SE) of four determinations with a Millipore ERS apparatus. (ii) Polarized uptake of methionine (>10:1 basal to apical ratio for all three lines), measured as described previously [J. Balcarova-Stander, S. E. Pfeiffer, S. D. Fuller, K. Simons, *EMBO J.* **3**, 2687 (1984)]. (iii) Polarized secretion of an endogenous sulfated glycoprotein [J. Urban, K. Parczyk, A. Leutz, M. Kayne, C. Kondor-Koch, *J. Cell Biol.* **105**, 2735 (1987)] was measured as described. The ratio of apical to basal secretion of the protein was 4.4 ± 0.6 for wild-type, 4.7 ± 0.9 for Ala^{664}, and 4.05 ± 0.9 for Asp^{664} receptors. The results presented here are for one representative clone of each type. To guard against clonal variation we also analyzed two other independent clones of each mutant. In all of the experiments described here (Figs. 1 to 4) similar results were obtained for all three clones of each mutant.
15. K. S. Matlin and K. Simons, *J. Cell Biol.* **99**, 2131 (1984).
16. K. E. Mostov, A. de Bruyn Kops, D. L. Deitcher, *Cell* **47**, 359 (1986).
17. P. P. Breitfeld, J. M. Harris, K. M. Mostov, *J. Cell Biol.* **109**, 475 (1989).
18. J. Casanova and K. Mostov, unpublished data.
19. M. S. Brown, R. G. W. Anderson, J. L. Goldstein, *Cell* **32**, 663 (1983).
20. Dissociation of Fab from the basal surface at 37°C was measured in minimum essential medium–bovine serum albumin (MEM-BSA) (*p*H 7.4) containing 0.5*M* sucrose to block internalization [J. E. Heuser and R. G. W. Anderson, *ibid.* **108**, 389 (1989)]. Dissociation at acidic *p*H was measured in MEM-BSA containing 10 m*M* acetic acid (*p*H 5.3), which also blocks internalization [J. E. Heuser, *ibid.* **108**, 401 (1989)]. Measurements were made at 1, 2.5, 5, 10, and 20 min from triplicate filters. Dissociation curves were superimposable for all three receptor forms.
21. M. P. Lisanti *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9557 (1988).
22. E. S. Sztul, K. E. Howell, G. E. Palade, *J. Cell Biol.* **100**, 1255 (1985).
22. G. D. Raphael, H. M. Druce, J. N. Baraniuk, M. A. Kaliner, *Am. Rev. Respir. Dis.* **138**, 413 (1988).
23. J. R. Bartles, H. M. Ferraci, B. Stieger, A. L. Hubbard, *J. Cell Biol.* **105**, 1241 (1987).
24. E. Maratos-Flier, C.-Y. Y. Kao, E. M. Verdin, G. L. King, *ibid.*, p. 1595.
25. S. Moktari *et al.*, *J. Membr. Biol.* **89**, 53 (1986); D. Massey *et al.*, *ibid.* **96**, 19 (1987); K. Matter, M. Brauchbar, K. Bucher, H.-P. Hauri, *Cell* **60**, 429 (1990).
26. J. Coloma and H. C. Pitot, *Nucl. Acids Res.* **14**, 1393 (1986); W. Hunziker, M. Spiess, G. Semenza, H. Lodish, *Cell* **46**, 227 (1986).
27. R. Rodewald and J.-P. Kraehenbuhl, *J. Cell Biol.* **99**, 159s (1984); N. E. Simister and K. E. Mostov, *Nature* **337**, 184 (1989).
28. W. Hunziker and I. Mellman, *J. Cell Biol.* **109**, 3291 (1989).
29. Confluent 35-mm dishes of cells were washed twice with tris-buffered saline and labeled with [^{32}P]P_i at 1 mCi/ml (Du Pont, Biotechnology Systems; 9000 Ci/mmol, HCl-free) for 3 hours in phosphate-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% dialyzed fetal bovine serum (FBS).
30. J. Houmard and G. R. Drapeau, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3506 (1972).
31. T. A. Kunkel, *ibid.* **82**, 488 (1985).

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Protease Nexin-II (Amyloid β-Protein Precursor): A Platelet α-Granule Protein

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Protease nexin-II (PN-II) [amyloid β-protein precursor (APP)] and the amyloid β-protein are major constituents of neuritic plaques and cerebrovascular deposits in individuals with Alzheimer's disease and Down syndrome. Both the brain and the circulation have been implicated as sources of these molecules, although they have not been detected in blood. Human platelets have now been found to contain relatively large amounts of PN-II/APP. Platelet PN-II/APP was localized in platelet α-granules and was secreted upon platelet activation. Because PN-II/APP is a potent protease inhibitor and possesses growth factor activity, these results implicate PN-II/APP in wound repair. In certain disease states, alterations in platelet release and processing and clearance of PN-II/APP and its derived fragments could lead to pathological accumulation of these proteins.

THE AMYLOID β-PROTEIN IS A 4.2-kD peptide that is deposited in neuritic plaques and the cerebrovasculature in Alzheimer's disease, Down syndrome, and to a lesser extent in normal aging (*1*). It is derived from a large precursor protein, the amyloid β-protein precursor (APP) (*2*), which can be translated from at least three alternatively spliced mRNAs, two of which contain an insert encoding a Kunitz-type protease inhibitor domain (*3*). The secreted form of APP containing the Kunitz-type inhibitor domain is protease nexin-II (PN-II) (*4, 5*). PN-II effectively inhibits chymotrypsin and trypsin (*4, 6*). In addition, it inhibits two proteases associated with growth factors: the epidermal growth factor binding protein (EGF BP) and the γ subunit of nerve growth factor (*6, 7*). The proteolytic events that lead to the formation and deposition of the amyloid β-protein

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