The basolateral sorting signal of the polymeric immunoglobulin receptor contains two functional domains

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SUMMARY

Basolateral sorting of the polymeric immunoglobulin receptor (pIgR) expressed in Madin-Darby canine kidney (MDCK) cells is mediated by a 17-residue sorting signal that resides in the cytoplasmic domain. We have recently analyzed the sequence requirements of the signal by alanine scanning mutagenesis. We found that basolateral sorting is mediated primarily by three amino acids: H656, R657 and V660. Individual mutations of each of these residues to Ala caused a substantial decrease in basolateral sorting and a corresponding increase in targeting to the apical surface. Structural analysis of 17-residue peptides corresponding to the signal revealed that V660 is in a β-turn (probably type I) secondary structure, and its mutation to Ala destabilized the turn. H656 and R657 were not part of the turn and substitution of Arg657 to Ala had no effect on the turn stability. These results suggested that the signal is comprised of two structurally distinct domains: a critical V660 in the context of the β-turn and an additional two residues (H656 and R657) that are not in the turn and probably are unimportant for its stability. Here we provide evidence suggesting that the two domains

are distinguishable not only by their structure but also by their function. Basolateral targeting of pIgR mutants bearing Ala mutations at either 656 or 657 was not affected by treatment with brefeldin A (BFA), while basolateral targeting of pIgR containing an Ala substitution at position 660 was markedly and uniquely stimulated by BFA. Compared to single Ala substitutions, simultaneous mutations of H656 and R657 to Ala caused an additional minor effect on basolateral and apical sorting, whereas double mutations of V660 and either H656 or R657 resulted in a maximal decrease in basolateral targeting and corresponding increase in apical targeting. These results suggest the existence of two domains in the signal. When both domains are destroyed, basolateral targeting is maximally inhibited. The results also imply that V660 mediates basolateral sorting by a different mechanism from H656 and R657. We suggest that V660 and perhaps more generally the β-turn may interact with BFA-sensitive adaptor complexes.

Key words: Brefeldin A, Trans-Golgi network, β-turn

INTRODUCTION

The plasma membrane of polarized epithelial cells consists of two domains, apical and basolateral, which have very different protein and lipid compositions (Mostov et al., 1992; Mostov and Cardone, 1995; Rodriguez-Boulan and Powell, 1992). Newly made plasma membrane proteins can be sent from the trans-Golgi network (TGN) directly to the basolateral or apical surface. Alternatively, proteins can be first sent to the basolateral surface. From that surface selected proteins are endocytosed and are either transcytosed to the apical surface, or recycled to the basolateral surface. Recent studies have shown that numerous basolateral proteins contain basolateral sorting signals, which direct the protein from the TGN to the basolateral surface. Generally, these signals consist of a short segment of the cytoplasmic domain and contain information that is necessary and sufficient for basolateral targeting. Analyzing the structure and function of these signals is a central issue in understanding the molecular mechanisms underlying cell polarity and membrane traffic.

The first basolateral signal to be identified was in the polymeric immunoglobulin receptor (pIgR) (Casanova et al., 1991b). This signal has been an extremely informative model for analyzing polarized sorting. The pIgR is targeted first to the basolateral surface and is then endocytosed and transcytosed to the apical surface. The cytoplasmic domain of the pIgR consists of 103 amino acids at the COOH terminus of the protein. The membrane proximal 17 residues of this domain (amino acids 653-669) are necessary for basolateral targeting. More importantly, transplantation of this signal to a heterologous apical reporter protein is sufficient to redirect the chimeric protein to the basolateral surface. This basolateral signal functions not only to direct the pIgR from the TGN to the basolateral surface, but also serves after endocytosis to recycle the pIgR back to the basolateral surface (Aroeti and Mostov, 1994). A similar conclusion has been reached in studies on the two basolateral sorting signals in the cytoplasmic domain of the low density lipoprotein receptor (LDLR) (Matter et al., 1993).

Ala scanning mutagenesis of the pIgR's 17-residue basolateral signal has shown that three amino acids are of primary importance to this signal (Aroeti et al., 1993). Replacement of the individual residues H656, R657, or V660 by Ala resulted in a marked (but not total) decrease in TGN-to-basolateral targeting and a corresponding increase in TGN-to-apical targeting. We used two-dimensional nuclear magnetic resonance spectroscopy to determine the structure in solution of 17-residue synthetic peptides corresponding to this signal. Residues R658-N-V-D661 have a propensity to adopt a β-turn (probably type I) in solution, while residues COOH-terminal to the turn seem to take up a nascent helix structure. We were unable to determine the secondary structure of residues 653-657. Substitution of V660 with Ala destabilizes the turn, while mutation of R657 to Ala did not appear to affect the turn. Although we have no direct data on the structure of this signal in the context of the native pIgR, these peptide model data are a useful starting point for investigating the structure and function of the signal. The finding of a type 1 β-turn in the signal is thought-provoking, as type 1 β turns are also important components of signals involved in clustering of receptors into clathrin coated pits at the plasma membrane. We were particularly struck by the finding that one of the three crucial residues, V660, was in the turn and essential for its structure, while the other two crucial residues, H656 and R657, were not in the turn and at least R657 appeared to be unimportant for the structure of the turn. This has raised the possibility that the signal consisted of at least two distinct domains. We hypothesize that domain I contains H656 and R657. Domain II is hypothesized to contain V660, and more generally might consist of the β-turn.

Our basic strategy in this paper was to determine if Ala mutations in domain I (i.e. H656 or R657) behaved differently from an Ala mutation of domain II (i.e. V660). If they behaved differently, this would support the hypothesis of two domains that are distinguishable not only by their structure, but also by their function. We used two experimental approaches. Our first approach was to examine the effects of brefeldin A (BFA) on sorting from the TGN to the basolateral surface of the various mutant pIgRs (Apodaca et al., 1993). Our second approach was to make pairwise double point mutations to Ala of the three crucial residues in the sorting signal.

MATERIALS AND METHODS

Cells and cell culture

MDCK cells (strain II) expressing the wild-type or mutant receptors were maintained for up to fifteen passages in minimal essential medium (MEM, Fisher) supplemented with 5% (v/v) fetal bovine serum (Hyclone), 100 U/ml penicillin, and 0.1 mg/ml streptomycin in 5% CO_2, 95% air. For all experiments, cells were cultured on 0.4 μm polycarbonate Transwell filters (12 mm diameter, Costar) as described, and used 3-4 days post culture. Fresh medium was added every other day after plating onto filters.

Site-directed mutagenesis and transfection

The construction of single Ala substitutions of either H656, R657, or V660 in receptors containing an intact cytoplasmic tail has been described previously (see Aroeti et al., 1993, and Fig. 1). The double Ala mutations were generated by the polymerase chain reaction (PCR) (for pIgR mutant nomenclature see Fig. 1). pIgR cDNA containing the Val660 to Ala mutation served as DNA template for making two different double mutations: pIgR H656,V660-AA and pIgR

R657,V660-AA, while pIgR cDNA containing the Arg657 to Ala mutation served as DNA template for constructing the pIgR H656,R657-AA double mutation. Both cDNA templates were subcloned into the *Bgl*II site of the vector pCB6. A DNA fragment (Fragment 1) encompassing nucleotides 1,828 to 2,142 in the pIgR cDNA was produced by PCR amplification using Primer 1: 5′GTAC-CTGTCGACCCAGCCAAGG3′ and Primer 2: 3′CGCCCCCG-GTACCGGCACCGGTATCGAGCTCGGGCC5′. Primer 2 contains a unique *Xho*I site. The *Xho*I site is introduced into position 2,132 of pIgR cDNA by mutating the native GT sequence in Primer 2 to AG (marked with underline). This silent mutation did not alter the amino acid composition of the pIgR. Fragment 1 was digested with *Sac*II (cuts at position 1,885 in the cDNA) and *Xho*I, resolved by electrophoresis and purified from agarose gel by GeneClean (Bio101). Primer 3: 5′GCCATAGCTCGAGCCCGGGCCAGGAG-GAACGCGGACCGAGTTTCC, primer 4: 5′GCCATAGCTC-GAGCCCGGCACGCGAGGAACGCGGACCGAGTTTCCATCGG and primer 5: 5′GCCATAGCTCGAGCCCGGGCCGCGAG-GAACGTGGACCGAGTTTCC were used to produce the pIgR mutants pIgR H656,V660AA, pIgR R657,V660-AA and pIgR H656,R657-AA, respectively. Codons encoding Ala are in bold and underlined. Underlined TC indicates the *Xho*I site described before. The sequence of Primer 6: 3′CGGACGTCCAGCTGAGATCTCCTA corresponds to the polylinker site of pCB6 which contains a unique *Cla*I site located downstream to the 3′ end of the cloned pIgR cDNA. A second DNA fragment (Fragment 2) was produced by PCR amplification using primer 6 and either primer 3, 4 or 5. Fragment 2 was digested with *Xho*I and *Cla*I, purified, and ligated together with Fragments 1 into pCB6 that has been previously digested with *Sac*II and *Cla*I. Deep Vent polymerase (New England Biolabs) was utilized in all PCR reactions. All PCR produced DNA sequences were confirmed by dideoxy sequencing.

Large scale DNA preparations (Qiagen) were made of all plasmids. Plasmid DNAs were then transfected into MDCK cells using the calcium phosphate precipitation procedure. Clones resistant to G418 (Gibco) were isolated, tested for stable expression, expanded, and their polarity was characterized as described earlier. All cell lines were similarly polarized. Experiments were performed on cells after 2-15 passages after cloning. Two or three different cell clones expressing each mutant receptor were analyzed and gave consistent results. The expression levels among all of the clones utilized in this report were roughly similar.

Protease-based assay for cell surface delivery

A protease-based delivery assay was applied to determine quantita-
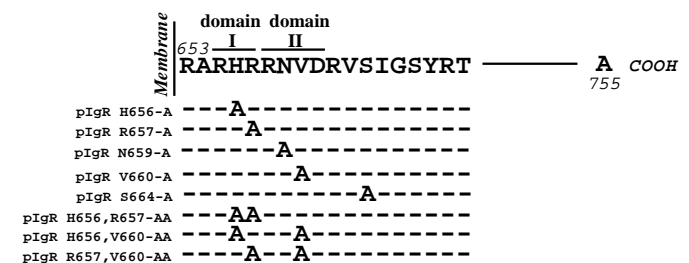
Fig. 1. Single and double Ala substitutions in the basolateral sorting signal. The 17-residue sequence of the basolateral sorting signal (residues 653-669) of the pIgR is presented in the single letter code. The full-length cytoplasmic tail continues to the C terminus with alanine 755 as the C-terminal residue. All Ala substitutions were constructed in the context of the full-length tail. The nomenclature of the single and double Ala substitutions in the two putative domains (domains I and II) of the signal are indicated. Mutations were constructed using M13 or PCR and mutant pIgRs were stably expressed in MDCK cells as described in Material and Methods.

tively the fraction of newly made receptors targeted to either the apical or basolateral surface. The procedure was described in detail in a previous report (Aroeti et al., 1993). Filter-grown cells were washed with warm (37°C) PBS^{++} (PBS containing 0.5 mM MgCl$_2$ and 0.9 mM CaCl$_2$) and starved for 15 minutes at 37°C in Dulbecco's modified Eagle's medium (DMEM) lacking Cys, but supplemented with Hanks' balanced salts and 20 mM Hepes, pH 7.4, 5% dialyzed fetal bovine serum. Cells were then pulse labeled for 15 minutes in the same medium containing ~3 mCi/ml [^{35}S]cysteine (1130 Ci/mmol; Du Pont-New England Nuclear). (In some experiments cells were incubated at 18°C for 2 hours to accumulate pIgR in the TGN.) Cells were subsequently chased for 60 minutes at 37°C in MEM/BSA (MEM containing Hanks' balanced salt solution, 20 mM Hepes, pH 7.4, 0.6% BSA) in the presence or absence of V8 protease (Boehringer) added to the basolateral medium. In cases where cells were treated with BFA, 10 µg/ml BFA (EpiCenter, stored as a 10 mg/ml stock solution in DMSO at −20°C), was included into all starvation, pulse and chase media. BFA non-treated cells were exposed to the same amounts of DMSO. At the end of the chase period, the apical and basolateral media were collected and the filters were washed three times with ice-cold MEM-BSA containing 10% horse serum (Sigma). SC was immunoprecipitated from both the apical and basolateral media of protease non-treated cells, and from the apical medium of protease-treated cells. Intact pIgR was immunoprecipitated from cells on filters as described. Radiolabeled pIgR and SC were resolved by SDS-PAGE, and radioactivity levels were determined by a PhosphorImager (Molecular Dynamics), using the Image-Quant software. The total amount of radiolabeled pIgR is derived by summing the amount of SC immunoprecipitated from the apical and basolateral media and the amount of intact pIgR immunoprecipitated from cells. In V8-treated cells, radiolabeled pIgR that has reached the basolateral surface is immediately digested by the protease and is no longer recognized by the immunoprecipitating antibodies. Consequently, the amount of total pIgR immunoprecipitated is reduced. The percentage of basolaterally targeted pIgR is derived from the reduced signal using the equation: % basolateral targeting = 1−(the amount of SC in the apical medium and pIgR present in V8-treated cells/the total amount of radiolabeled pIgR and SC in protease non-treated cells)×100.

Upon arrival at the apical surface, the pIgR is efficiently cleaved by an endogenous protease to SC. The SC is released to the apical medium. As all cysteines except one reside in the SC fragment, the amount of apically released SC in cells treated with basolateral V8 directly reflects the amount of pIgR molecules delivered to the apical surface. The percentage of direct apical delivery is derived from the equation: % apical targeting = (the amount of SC released at the apical medium following treatment with basolateral V8/the total amount of labeled pIgR in protease non-treated cells + SC)×100.

Biotinylation-based assay for apical targeting

To determine the amount of pIgR directed to the apical surface by an assay that does not utilize endogenous proteases, it is necessary to inhibit the activity of the endogenous protease that cleaves the pIgR to SC at the apical plasma membrane. This was achieved by pre-treating the monolayers with the protease inhibitor leupeptin (100 µg/ml, Chemicon) for 1 hour at 37°C. The protease inhibitor was present in the media bathing the cells throughout the entire assay. Cells were pulse labeled with [^{35}S]Cys for 10 minutes at 37°C and chased for 0-60 minutes with V8 protease present in the basolateral medium. Monolayers were either treated or not treated with 10 µg/ml BFA. The V8 protease immediately digests pIgR molecules that arrive at the basolateral surface, thus eliminating basolateral to apical transcytosis of the pIgR. The protease does not affect direct apical targeting of the pIgR. At the end of the chase, cells were washed extensively with ice-cold Hank's buffer lacking phosphate but containing 20 mM Na-Hepes, pH 7.4. Proteins at the apical surface were then biotinylated with 500 µg/ml NHS-LC-biotin (Pierce) on ice as

before. Following extensive washes with MEM/BSA supplemented with 50 mM glycine, cells were lysed in 2.5% Triton dilution buffer (2.5% Triton X-100, 100 mM NaCl, 5 mM EDTA, 100 U/ml trasylol, 0.1% NaN$_3$, 2 mM PMSF, 50 mM triethanolamine-HCl, pH 8.6) and pIgR was immunoprecipitated using sheep anti-SC antibodies coupled to agarose-Protein G beads (Pharmacia) as described. The bead suspension was divided into two fractions. One fifth of the immunobead suspension was processed for analysis by SDS-PAGE. This fraction was used to determine the total amount of radiolabeled pIgR. The rest of the immunobead suspension was briefly pelleted and boiled for 5 minutes in 20 µl of 10% SDS. Biotinylated pIgR was precipitated by streptavidin agarose, analyzed by 10% SDS-PAGE and radioactivity was quantified by a PhosphorImager as described (Aroeti et al., 1993).

RESULTS

Differential effects of BFA on mutations in the two domains

BFA has been a very useful tool for the dissection of a number of membrane traffic processes (Klausner et al., 1992). BFA has been shown to affect the polarized sorting of a variety of proteins. For instance, the basolateral transport of wild-type LDLR was reportedly reduced to about 35% upon treatment with BFA (Matter et al., 1993), while the basolateral sorting of uvomorulin was unaffected (Low et al., 1992). Apical sorting of membrane and secretory proteins is blocked by BFA (Low et al., 1991, 1992). BFA also causes a striking tubulation of the TGN in MDCK cells (Wagner et al., 1994). We have previously examined the effect of BFA on polarized sorting of the wild-type pIgR (Apodaca et al., 1993). We found that basolateral targeting of the pIgR was reduced to about 30% by 10 µg/ml BFA. Our earlier data also led to the suggestion that there might be multiple pathways for transport to the basolateral surface. In that study, BFA was shown primarily to affect targeting from the TGN to the cell surface. In the present study we were interested to determine if BFA would have differential effects on the polarized sorting of pIgRs containing Ala mutations in the structurally distinct domains of the basolateral sorting signal (Fig. 1). In the absence of BFA, approximately 80% of newly synthesized wild-type pIgR is delivered to the basolateral surface within one hour. Treatment with BFA results in an inhibition of basolateral delivery to about 35%, in agreement with previous work (Fig. 2A).

Ala point mutations whose basolateral deliveries in the absence of BFA are close to the 80% level of wild-type pIgR (i.e. pIgR N659-A and pIgR S664-A) behaved nearly identically to wild-type pIgR in response to BFA (Fig. 2A). More strikingly, even mutants whose basolateral delivery in the absence of BFA was only about 40% exhibited basolateral delivery in the range of 35-40% in the presence of BFA. (pIgR V660-A is a major exception, as discussed below.) Therefore, irrespective of whether basolateral delivery in the absence of BFA was 80% (wild-type pIgR, pIgR R659-A, pIgR 664-A) or approximately 45% (pIgR H656-A, pIgR R657-A), as a result of BFA treatment basolateral delivery was reduced to a value of approximately 35-40%. Much of the pIgR was therefore retained intracellularly in the presence of BFA, in complete agreement with our previous results. There were no statistically significant differences in basolateral delivery in the presence of BFA among the various mutants of pIgR (except pIgR V660-A). This value did not differ significantly upon treatment with
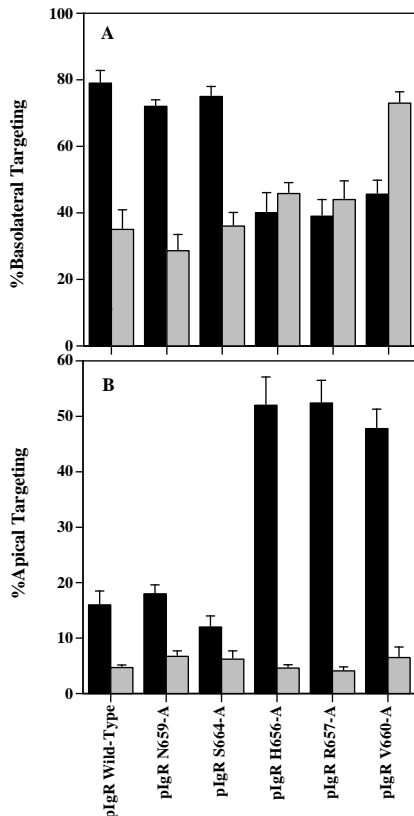
Fig. 2. Direct targeting of wild-type and Ala mutant pIgRs to the basolateral (A) or apical (B) surface in BFA-treated (stippled bars) and BFA-untreated (filled bars) MDCK cells. Direct targeting of the various receptors to the respective cell surface was measured over a 60 minute chase at 37°C using the protease sensitivity-based assay. In BFA-treated cells, BFA (10 μg/ml) was present in the media bathing both the apical and basolateral surfaces of the cells throughout the pulse and chase steps as indicated in Materials and Methods. Results are mean ± s.e. ($n \geq 13$).

higher BFA concentrations (up to ~20 μg/ml), or upon lengthening the chase time up to 90 minutes (data not shown).

The striking exception was pIgR V660-A. In the absence of BFA, about 45% of the newly synthesized molecules were delivered to the basolateral surface. However, in the presence of BFA, basolateral targeting was dramatically increased, to roughly the wild-type level of 75%. We also tried an alternative protocol in which the pIgR V660-A was accumulated in the TGN by chasing for 2 hours at 18°C, prior to adding BFA and chasing at 37°C (Aroeti et al., 1993; Apodaca et al., 1993). Based on five measurements, the result was 67±5% basolateral delivery and 10±2% apical delivery (data not shown).

It should be noted that our assay measures the susceptibility of the pIgR to protease added to the basolateral surface at 37°C. It is possible that the protease gains access to pIgR that has not actually reached the cell surface, but rather is in some intracellular compartment, including perhaps a compartment which is modified (e.g. tubulated) by the BFA treatment. Nevertheless, there is a striking difference between the behavior of pIgR V660-A, which is very susceptible to basolateral protease in the presence of BFA, and that of the wild-type pIgR and all other mutant pIgRs, where there is far less susceptibility to

basolateral protease in the presence of BFA. As far as we are aware, pIgR V660-A is the only plasma membrane protein that is known to respond to BFA in this manner, which suggests that its sorting is fundamentally different from that of other pIgR mutants and all other plasma membrane proteins examined so far.

For both the wild-type pIgR and all of the mutants tested (including pIgR V660-A), BFA dramatically reduced apical targeting to approximately 5% (Fig. 2B). This was irrespective of the fraction delivered to the apical surface in the absence of BFA. Our assay for delivery to the cell surface relies on the efficient cleavage of the pIgR by an endogenous protease at the apical surface. We considered the possibility that delivery of this protease to the apical surface might be inhibited by BFA, resulting in an artificially low figure for apical delivery. To circumvent this possible problem we employed a surface biotinylation procedure that is independent of proteolysis to measure direct apical targeting of the pIgR. As shown in Fig. 3, BFA strikingly blocked the delivery of pIgR R657-A to the apical surface. BFA had almost no effect on the relatively small fraction of apically targeted wild-type receptor.

Double mutations functionally distinguish the two domains

Our second approach to examine the existence of distinct domains in the basolateral targeting signal was to make pairwise double mutations to Ala of the three crucial residues (i.e. pIgR H656,R657-AA; pIgR H656,V660-AA, and pIgR R657, V660-AA). We expected to obtain a greater inhibition of basolateral sorting and an increase in apical sorting when both structural domains are destroyed and a smaller effect when each of the domains are singly mutated. We found that simultaneous mutation of both H656 and R657 to Ala had only a slightly greater and statistically insignificant effect on basolateral sorting than any single point mutation. (The polarized delivery data are presented in Table 1, and the degree of sta-
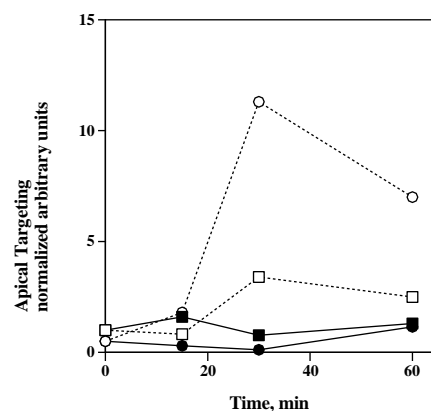
Fig. 3. Direct targeting of wild-type pIgR (□,■) and pIgR R657-A (○,●) to the apical surface measured by cell surface biotinylation. Direct apical targeting of newly synthesized receptors to the apical surface was determined by pulse chase and surface biotinylation as described in Materials and Methods. BFA treatment was performed as in Fig. 2 (□,○, −BFA; ■,●, +BFA). The results represent the mean values of two experiments each of which performed in duplicates. The error bars are smaller than the symbol size. The amount of surface recovered pIgR was normalized to the total amount of immunoprecipitated receptors recovered in all fractions.

Table 1. Effect of double point mutations on polarized targeting of pIgR

Group no.	pIgR mutant	Apical targeting ± s.e. (%)	Basolateral targeting ± s.e. (%)	N
1	pIgR H656R657-AA	56±4	36±4	10
2	pIgR H656V660-AA	70±5	22±5	12
3	pIgR R657V660-AA	71±5	21±5	12
4	pIgR H656-A	52±5	40±6	13
5	pIgR R657-A	52±4	39±5	13
6	pIgR V660-A	48±4	45±4	13

Double point mutations to Ala at positions H656, R657, and V660 were constructed and the polarity of pIgR targeting was analyzed as described in Materials and Methods. For comparison, data on the single point mutations to Ala (Aroeti et al., 1993) are also presented. The number of independent measurements (N) is indicated.

tistical significance in pairwise comparisons of each double mutant with each single point mutant is given in Table 2.) In contrast, simultaneous mutations of V660 and either H656 or R657 caused a sizable, statistically significant decrease in basolateral sorting and a corresponding increase in apical sorting compared to single point mutations of H656, R657, or V660, or to the double mutant of H656 and R657. Stated slightly differently, all of the mutants can be divided into two categories. Simultaneous mutation of V660 and either H656 or R657 produced apical targeting in the range of 70-71% and basolateral targeting of 21-22%. Mutation of any single residue, or the H656,R657 double mutant, produced apical targeting of 48-56% and basolateral targeting of 36-45%. Values within each of the two categories were statistically indistinguishable, while differences between the two categories were statistically highly significant. These data further

Table 2. The statistical significance of differences in apical and basolateral targeting between each of the mutants

Apical targeting: probability that difference is statistical only (P)	Basolateral targeting: probability that difference is statistical only (P)
P(1, 4)>0.25	P(1, 4)>0.25
P(1, 5)>0.1	P(1, 5)>0.25
P(1, 6)>0.05	P(1, 6)>0.05
P(2, 4)<0.01	P(2, 4)~0.01
P(2, 5)~0.005	P(2, 5)~0.01
P(2, 6)<0.001	P(2, 6)<0.001
P(3, 4)<0.01	P(3, 4)~0.01
P(3, 5)<0.005	P(3, 5)<0.01
P(3, 6)<0.001	P(3, 6)<0.001
P(1, 2)~0.01	P(1, 2)~0.01
P(1, 3)~0.01	P(1, 3)~0.01
P(2, 3)>0.25	P(2, 3)>0.25

This analysis was performed using Student's *t*-test. Comparison of each of the double mutants to each of the single mutants is given above the double line, while comparison of the double mutants to each other is given below the double line. The groups compared are indicated in parenthesis. The *P* value is the probability that a given difference could be due to chance alone. Conventionally, a *P* value of less than 0.05 (meaning that there is a l in 20 chance that the difference is due to chance) is taken to indicate that the difference is statistically significant. By this criteria, the differences between pIgR H656V660-AA or R657V660-AA and any of the other mutants is statistically significant, but none of the differences between any of the other mutants is significant.

support our hypothesis that the basolateral signal contains two distinct domains.

Effect of BFA on the double mutants

Finally, we examined the effects of BFA on polarized sorting of the three double point mutants (Fig. 4). Both double mutants that contained the V660-A change (i.e. pIgR H656,V660-AA and pIgR R657,V660-AA) responded to BFA by showing a roughly twofold increase in basolateral targeting, to a level of approximately 40-45%. In contrast, the pIgR H656,R657-AA double mutant did not show a statistically significant increase in basolateral targeting in response to BFA. There are two possible interpretations to this result. First, in the presence of BFA all of the single and double point mutants (other than the V660-A single mutations) gave a level of approximately 40-45% basolateral targeting and there were no statistically significant variations between mutants. This might imply that the V660-A single mutation is truly unique, and that its unique response to BFA (whose mechanism is unknown) is abrogated by introduction of a second mutation at H656 or R657. Alternatively, one might consider that the amount of basolateral
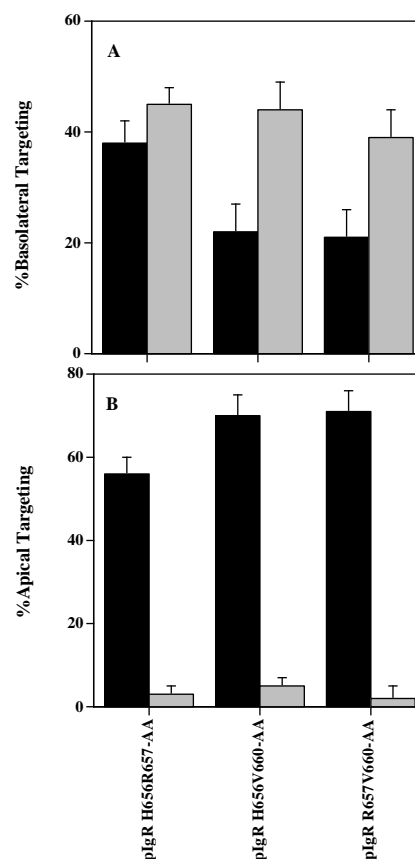
Fig. 4. Direct targeting of double mutant pIgRs to the basolateral (A) or apical (B) surface in BFA-treated (stippled bars) and BFA-untreated (filled bars) MDCK cells. Direct targeting of the various double mutant receptors to the respective cell surface was measured over a 60 minute chase at 37°C using the protease sensitivity-based assay. In BFA-treated cells, BFA (10 μg/ml) was present in the media bathing both the apical and basolateral surfaces of the cells throughout the pulse and chase steps as indicated in Materials and Methods. Results are mean ± s.e. (*n*≥13).

targeting of these two double mutants in the absence of BFA was somewhat lower than that of any single mutant, so that the absolute amount of the increase in basolateral sorting induced by BFA was smaller than that seen with the pIgR V660-A single mutant. From this view, the behavior of the double mutants suggests that as long as one of the mutated sites is V660-A, then BFA will cause a stimulation in basolateral targeting. Irrespective of which interpretation is correct, all of our data is consistent with the hypothesis that the V660-A mutation responds to BFA in a manner which is different from either the wild-type pIgR or mutations at other sites.

DISCUSSION

The basolateral sorting signal of the pIgR has been studied in considerable depth, and it is now perhaps the best understood basolateral signal. The pIgR signal was the first to be shown to be both necessary and sufficient for basolateral sorting, criteria which have been formally met for only a few other basolateral signals (Casanova et al., 1991a,b; Matter et al., 1993; Thomas et al., 1993). The pIgR signal has been systematically mutated by Ala scanning, progressive truncation, and other types of mutations. Most importantly, the three-dimensional structure of a synthetic peptide corresponding to the signal has been determined. This structure has indicated that, like signals for endocytosis via clathrin coated pits, the pIgR basolateral signal contains a type 1 β turn. This has led to the suggestion that type 1 β turns may be the conserved feature of both basolateral signals and clathrin-mediated endocytosis signals, and perhaps other types of sorting signals. Therefore, dissecting the pIgR basolateral signal may give insights into the nature of basolateral signals, and perhaps sorting signals in general.

Taken together with our previous work, there are now multiple lines of evidence that the basolateral signal of the pIgR consists of two structurally and functionally distinct domains. Of the three residues that are most important for basolateral sorting, V660 is part of a type 1 β turn, whereas H656 and R657 are outside of the turn (Aroeti et al., 1993). Unlike wild-type pIgR, pIgR mutants pIgR H656-A and pIgR R657-A (and similarly wild-type and mutant LDLR) which adopt a uniform 35% basolateral delivery in the presence of BFA, pIgR V660-A strikingly increases its basolateral sorting to 75%. Simultaneous mutation of H656 and R657 to Ala only slightly alters polarized sorting compared to either single mutation. In contrast, simultaneous mutation to Ala of either H656 or R657 together with V660 produces a statistically significant greater inhibition of basolateral sorting and stimulation of apical sorting compared to any single point mutation. These data greatly refine our understanding of the structure and function of this basolateral sorting signal.

It will be interesting to determine if other basolateral signals (and other categories of sorting signals) have a two domain structure. The two basolateral sorting signals in the LDLR require both a tyrosine and a cluster of acidic residues located some distance towards the carboxy terminus (Matter et al., 1992, 1994). However, the types of analysis that we have performed to demonstrate the existence of two distinct domains in the pIgR basolateral sorting signal have not been reported for the LDLR basolateral signals, and so the presence of two distinguishable domains in the two LDLR basolateral signals is unresolved.

The simplest interpretation of our results with the double mutations is that H656 and R657 are both in the same domain, so this double mutation cannot damage this domain much further than either single mutation. In contrast, V660 appears to be in a domain that is distinct from H656 and R657, so mutation of two residues in different domains (i.e. H656,V660-AA, or R657, V660-AA) has a greater effect on basolateral sorting. We have previously described a mutant pIgR in which 15 of the 17 residues comprising the entire basolateral signal have been deleted, and the remaining 88 carboxy-terminal residues of the pIgR fused on in frame (Casanova et al., 1991a,b). The polarized sorting of this deletion mutant was quantitatively very similar to the H656,V660-AA and R657, V660-AA double mutants. This suggests that these double point mutations introduced resulted in nearly complete destruction of the basolateral signal, and that there are no other domains in the pIgR that are capable of independent function in basolateral sorting.

We do not know precisely how BFA affects polarized sorting. Nevertheless, as in other systems, BFA is a useful tool to dissect membrane traffic processes (Klausner et al., 1992). In particular, a differential response to BFA of two molecules that are potentially sorted by the same mechanism can be used to indicate the presence of underlying differences in the sorting mechanisms used by those molecules. In the present study, we found that BFA had differential effects on different mutant pIgRs. BFA treatment results in a uniform 35-40% basolateral sorting of almost every protein so far examined, including most mutants of pIgR and LDLR. The pIgR V660-A exhibited unique behavior, in that in the presence of BFA, its basolateral sorting was increased to 75%. The anomalous behavior of the pIgR V660-A is good evidence that its sorting mechanism somehow differs from other proteins, and further supports the concept that the basolateral sorting signal of the pIgR contains two domains. A differential effect of BFA on another pathway of delivery to the apical surface, i.e. transcytosis from the basolateral to the apical surface, has previously been observed. Transcytosis of IgA bound to the pIgR is strongly inhibited by BFA. However, transcytosis of pIgR that does not have IgA bound is not inhibited by BFA, and transcytosis of ricin, horseradish peroxidase, and transferrin receptor to the apical surface is actually stimulated by BFA (Matter et al., 1993; Prydz et al., 1992). We do not know if these differential effects of BFA on transcytosis are related to the effects of BFA on apical or basolateral delivery of various mutants of pIgR that we now describe, but the possibility merits further investigation.

BFA is known to block the binding of at least three coat proteins: coatomer involved in ER-Golgi transport, the TGN clathrin vesicle adaptor AP1 involved sorting of the mannose-6-phosphate receptor to endosomes, and the P200 Golgi coat protein. Coat proteins involved in polarized sorting have not been definitively identified, but P200 is a likely candidate for a coat involved in TGN-to-basolateral sorting (Narula et al., 1992). By analogy to the AP2 adaptor of plasma membrane clathrin coated pits that is believed to interact with internalization signals containing a Tyr residue in a β-turn, it seems plausible that the Val in the β-turn of the pIgR basolateral sorting signal is recognized by a coat protein that mediates basolateral sorting in the TGN. We do not know why the

mutant pIgR V660-A has a unique response to BFA, but this result suggests that the mutant receptor somehow utilizes a different pathway and/or molecular mechanism to reach the cell surface than most other molecules. Considering that the pIgR basolateral signal apparently consists of two domains, we can further imagine that each domain interacts with a different protein or complex involved in basolateral sorting. Perhaps the V660-A mutant has uncovered or stimulated a mechanism and/or pathway that is BFA-insensitive or even BFA-augmented. For instance, it has previously been suggested that transport of the pIgR and other proteins to the basolateral surface may be via multiple routes, e.g. one path might be via endosomes, whereas the other could be direct. BFA may differentially inhibit or stimulate these pathways, which could account for the variable effect of BFA on basolateral targeting of different proteins, such as the LDLR and uvomorulin (Low et al., 1992; Matter et al., 1993), as well as the partial inhibition of basolateral delivery of wild-type pIgR by BFA. The V660-A mutant could have a singular ability to utilize (or avoid) one of these pathways in the presence of BFA. Our results suggest that the V660-A mutant may be a uniquely powerful tool to dissect these multiple pathways and the factors that control access into them.

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