The Cytoplasmic Domains of a $\beta_1$ Integrin Mediate Polarization in Madin-Darby Canine Kidney Cells by Selective Basolateral Stabilization*

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In Madin-Darby canine kidney cells, newly synthesized apical and basolateral membrane proteins are generally transported directly to their respective cell surface domain due to targeting determinants that mediate sorting in the Golgi complex. In several basolateral membrane proteins, these targeting determinants reside in the cytoplasmic domains. We show here that basolateral expression of the human $\alpha_5\beta_1$ integrin in stably transfected Madin-Darby canine kidney cells is also mediated by the cytoplasmic domains. Distinct regions in both cytoplasmic domains were found to be sufficient to mediate basolateral expression independently from one another. Unexpectedly, newly synthesized wild-type $\alpha_5\beta_1$ and basolaterally expressed chimeras containing the cytoplasmic domain of either $\alpha_5$ or $\beta_1$ were integrated into both cell surface domains, preferentially apically, during biosynthesis. The apical pools of wild-type integrin and chimeric subunits were found to become quickly degraded, whereas the basolateral pools were stabilized. Thus, the cytoplasmic domains of the $\alpha_5\beta_1$ integrin are independently sufficient to mediate sorting by selective basolateral stabilization.

The plasma membrane of polarized epithelial cells is divided into two morphologically, functionally, and biochemically distinct cell surface domains. The maintenance of cell surface polarity requires the continuous sorting of newly synthesized and internalized membrane components. Once arrived at the trans-side of the Golgi apparatus, newly synthesized membrane proteins are sorted into cell surface transport pathways in a manner that can vary from one epithelial tissue to another and from one protein to another (1–4).

With one exception, all studied apical and basolateral membrane proteins are directly sorted to their respective cell surface domain in the Golgi apparatus in MDCK cells. Apical sorting can be mediated by different types of determinants including transmembrane domains and glycosylphosphatidylinositol anchors as well as luminal N-linked and perhaps O-linked carbohydrates (5–10). Basolateral targeting has been associated with cytoplasmic domain determinants that mediate direct basolateral transport in the Golgi apparatus as well as in endosomes (2, 3). Membrane proteins lacking apical and basolateral targeting determinants accumulate in the Golgi apparatus, indicating that efficient cell surface transport does not occur by default (10).

In one clone of MDCK cells, polarization of Na$^+$ K$^+$-ATPase is mediated by selective basolateral stabilization after delivery to both cell surface domains (11). The apical pool of Na$^+$ K$^+$-ATPase does not become transcytosed but is degraded, as seen during the repolarization of MDCK cells in calcium switch experiments (12). Although nonpolarized cell surface transport occurs only in a mutant MDCK cell line deficient in glycolipid sorting, the steady-state polarity of Na$^+$ K$^+$-ATPase seems to be enhanced by selective basolateral stabilization also in wild-type MDCK cells (13, 14).

Integrins are functionally important basolateral membrane proteins composed of an $\alpha$ subunit and a $\beta$ subunit (15, 16). MDCK cells express a variety of different $\alpha$ and $\beta$ subunits (17, 18). $\beta_1$ seems to be the predominant $\beta$ subunit and is involved in the formation of functional receptors for different extracellular matrix components as well as in the determination of the spatial orientation of polarized epithelial cells (18–20).

We studied basolateral sorting of the human $\alpha_5\beta_1$ integrin in stably transfected MDCK cells, which was basolaterally expressed in the steady state. The cytoplasmic domains of both subunits were found to be able to mediate the basolateral sorting of dimeric integrins as well as that of a chimeric reporter protein independently from one another but exhibited different capacities. Unlike other basolateral sorting signals, the presence of the cytoplasmic domains of $\alpha_5$ and/or $\beta_1$ did not result in direct basolateral targeting, but in stabilization of the basolateral pool after transport to both cell surface domains.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Mutagenesis, and Transfection—**MDCK strain II cells were grown in Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose) as described previously (21). For polarity experiments, the cells were cultured for 5–8 days on tissue culture-treated polycarbonate filters (Costar Transwells) with a pore size of 0.4 $\mu$m and a diameter of 24 mm (for biochemical assays) or 12 mm (for immunofluorescence).

The cDNAs coding for human $\alpha_5$ and $\beta_1$ (generously provided by Dr. E. Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, CA) were cloned into Bluescript SK$^+$ and into the eukaryotic expression vectors pCB7 ($\alpha_5$; selectable with hygromycin) or pCB6 ($\beta_1$; selectable with G418). $\alpha_5$CT9 and $\beta_1$CT10 were constructed using the Bluescript constructs and PCR-based mutagenesis to introduce premature stop codons. Fc receptor chimeras were constructed as described previously using a modified FcRII-B2 cDNA coding for the apically expressed tail-minus receptor with a A/III site just before the stop codon (21). cDNA fragments corresponding to the cytoplasmic domain of $\alpha_5$ (amino acids 3–28, resulting in FcR-$\alpha_5$3-28) or $\beta_1$ (amino acids 11–47, resulting in FcR-$\beta_1$11-47) with a A/III and a XhoI site were synthesized and cloned into the Fc receptor cDNA in pCB6. These two cDNAs were then

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1 The abbreviations used are: MDCK, Madin-Darby canine kidney; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis.

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Sorting of Integrins in Epithelia

used to construct chimeras with modified cytoplasmic domains using primers with 5′ XhoI sites for cloning. All constructs were sequenced over the entire subcloned PCR-derived fragment by dyeodeoxy sequencing. MDCK cells were transfected and selected using G418 (Pc86) or hygromycin (pCB7), respectively (21). For double transfections, stable cell lines were generated expressing either wild-type or truncated human β1. These cell lines were then used to co-express α5, cDNAs. Single clones were picked and analyzed for expression by immunofluorescence. At least three clones and an uncloned cell line (derived from a pool of the nonpicked clones) were grown and analyzed for all transfections.

Immunofluorescence and Confocal Microscopy—Transfected cells were cultured on filters for 5–8 days and then fixed with or without an overnight preincubation with 10 mM sodium butyrate. To label chimeric Fc receptors with a polyclonal antibody (22) and human α5 with monoclonal antibody P1D6 (Life Technologies, Inc.), the cells were fixed for 20 min; to label human β1 with monoclonal antibody P4C10 (Life Technologies, Inc.), the cells were fixed for 60 min with 3% paraformaldehyde. The long fixation for the β1 staining eliminated background staining due to endogenous β1 if the primary antibody was sufficiently diluted (1:8000; each batch was newly titrated). The samples were then blocked and permeabilized with phosphate-buffered saline containing 0.1% saponin, 10 mM glycerin, 0.5% bovine serum albumin, and 0.05% sodium azide (23). The primary and secondary antibodies were diluted in the same solutions, and the incubations were performed for 60 min at room temperature. In some experiments, cell surface chimeras were labeled by incubating the unfixed cells with primary antibody diluted in phosphate-buffered saline containing 0.5% bovine serum albumin and 0.5 mM CaCl2 added from both sides of the filter culture for 2 h on ice (24). The samples were analyzed by confocal microscopy as described previously (22).

Both the α5 and the β1 Cytoplasmic Domain Contain Basolateral Sorting Information—Because basolateral sorting of several membrane proteins has been shown to depend on distinct cytoplasmic targeting determinants, we constructed C-terminally truncated α5 and β1 integrin subunits lacking most of the cytoplasmic domains (Fig. 2: α5CT9 and β1CT10) and generated stable cell lines co-expressing the two mutant subunits. Filter-grown cells preincubated overnight with sodium butyrate were fluorescence labeled using either the anti-α5 or the anti-β1 monoclonal antibody and analyzed by confocal microscopy. Fig. 1B shows that both antibodies efficiently stained the apical membrane of MDCK cells expressing human α5 and β1 integrin subunits lacking their cytoplasmic domains (α5CT9 and β1CT10), and only a little staining of the basolateral cell surface was detected. Thus, one or both of the cytoplasmic domains are required for basolateral expression of human α5β1 integrin in MDCK cells. Furthermore, the absence of cytoplasmic basolateral sorting information results in the preferential apical expression of the integrin, as in the case of many monomeric membrane proteins (2).

RESULTS

Human α5β1 Integrin Is Expressed at the Basolateral Cell Surface in Stably Transfected MDCK Cells—MDCK cells endogenously express several different integrins, but β1 seems to be the most prominent β subunit (17, 18). We therefore decided to use human β1 and α5 for the targeting studies; these two subunits can dimerize together, forming a receptor for fibronectin, and the human α5 subunit can easily be specifically detected because MDCK cells do not seem to express significant amounts of the α5 integrin subunit (17). To reconstitute this human integrin in MDCK cells, we first generated stable cell lines expressing human β1. This resulted in several clones that all expressed the human integrin subunit in the basolateral membrane (data not shown). We chose two homogeneously expressing clones into which we transfected a cDNA coding for human α5. Cell lines expressing the two human integrin subunits were then cultured on filters for 5 days to allow full polarization, and the polarity of expression was then studied by immunofluorescence combined with confocal microscopy. Because both the anti-α5 and anti-β1 antibodies are derived from mouse, we stained separate cultures for the two subunits.

Fig. 1A shows confocal sections obtained from monolayers preincubated overnight with sodium butyrate and then labeled with the two integrin antibodies. In both cases, very little apical staining could be detected, but the lateral and the basal plasma membranes were efficiently labeled. When nontransfected MDCK cells were labeled with the two antibodies under the same conditions, no staining could be detected (the anti-β1 antibody showed some staining in cells that were gently fixed for 15 min, but not if the cells were fixed as described here for 60 min). Basolateral expression of the two subunits was also obtained when they were separately transfected (data not shown). The cell surface expression in such single transfections was lower, and the β1 subunit could be detected accumulating in the endoplasmic reticulum, suggesting that the expression of only one subunit did not result in efficient cell surface transport due to insufficient amounts of partners for dimerization. Although this suggests that the two transfected subunits dimerized together, it could also be that at least a fraction of the transfected subunits was transported to the cell surface in association with an endogenous integrin subunit. As shown below, however, co-expressed subunits can determine the polarity of each other’s cell surface distribution, indicating that in the steady state, the vast majorities of co-transfected human integrin subunits must be dimerized with one another.
sufficient to mediate basolateral expression. Cells expressing wild-type β1 and α5CT9 were labeled by both antibodies on both cell surface domains (Fig. 1D), suggesting that the cytoplasmic domain of β1 also mediates basolateral expression, but at an apparently lower efficiency than the cytoplasmic domain of α5.

These experiments also demonstrated that one transfected subunit could efficiently redirect the other one, indicating that most of the integrin staining we observed must have been due to the transfected human α5 and β1 dimerized with each other. This is also supported by the observation that efficient apical expression was not observed when the cells were transfected with either one of the mutant subunits alone (data not shown).

It is important to note that expression of mutant integrin subunits did not negatively affect MDCK cells. We could not detect decreases in transepithelial electrical resistance, indicating that the transfected cells were still forming electrically tight monolayers, and the cells did not show any obvious morphological alterations when observed by electron microscopy after embedding in Epon and thin sectioning (data not shown).

Both Cytoplasmic Domains Are Independently Sufficient to Mediate Basolateral Expression—Because integrin affinity to the ligand can be affected by mutations in the cytoplasmic domains, it could be that the deletions of the cytoplasmic domains caused a redistribution due to the effects on substrate binding. The effects of mutations that we observed on the polarized cell surface distribution, however, do not correlate with the known effects on affinity (27). To test a direct involvement of the integrin cytoplasmic domains in basolateral sorting, we linked the entire cytoplasmic tail of α5 (amino acids 1–28) or the region of β1 required for basolateral expression of the integrin (amino acids 11–47) to the transmembrane and FIG. 1. Polarized expression of wild-type and C-terminally truncated human αβ integrin in MDCK cells. Stably transfected MDCK cells co-expressing the human α5 and β1 integrin subunits (A), α5CT9 and β1CT10 (B), wild-type α5 and β1CT10 (C), or α5CT9 and wild-type β1 (D) were plated on filters; after 1 week of culture and an overnight incubation with sodium butyrate, they were fixed and processed for immunofluorescence using either an anti-α5 (top) or anti-β1 (bottom) monoclonal antibody. The samples were analyzed with a confocal microscope. For each staining, an optical section derived from the apical region of the monolayer (apical), one from approximately the middle of the monolayer (lateral), and one taken close to the filter (basal) are shown. At least two different clones and an uncloned cell line were analyzed for each combination of the transfected subunits. Bars, 10 μm.

FIG. 2. Amino acid sequences of the cytoplasmic domains of wild-type, mutant, and chimeric human α5 and β1 integrin subunits. The cytoplasmic domain amino acid sequences of wild-type and mutant α5 and β1 (A) and of chimeras consisting of the ecto- and transmembrane domains of the mouse Fc receptor for IgG (FcRII) and α5 or β1 cytoplasmic domain sequences (B) are shown in the single-letter code. In A, the arrows indicate the positions of the C-terminal truncations (the number after CT indicates the number of residual cytosolic amino acids). In B, amino acids derived from either α5 or β1, respectively, are underlined, and the arrows indicate the position of amino acid substitutions (labeled with the code of the replaced and the introduced amino acids and the position in the cytoplasmic domain). For the different chimeric constructs of α5 (FcR-α5) and β1 (FcR-β1), the stretch of amino acids derived from α5 or β1, respectively, is indicated with a double-headed arrow and the number of the first and the last integrin-derived amino acid.
extractytoplasmic domain of the mouse Fc receptor for IgG (Fig. 2), a protein that is apically expressed in the absence of a basolateral sorting signal in its cytoplasmic domain (28, 21). We then generated stable MDCK cell lines expressing these chimeric constructs and analyzed polarized expression in filter-grown cells with an antibody recognizing the Fc receptor ectodomain.

Fig. 3 shows confocal sections taken from such samples derived from cells treated with (A and B) or without (C) sodium butyrate. The chimera containing the cytoplasmic domain of $\alpha_5$ (Fig. 3A) was basolaterally expressed, and only a little apical staining could be detected. The cytoplasmic domain of human $\alpha_5$ is thus sufficient to mediate basolateral expression of the reporter protein. When MDCK cells expressing the $\beta_1$ Fc receptor chimera were analyzed, labeling of both cell surface domains could be detected after the induction of high expression levels by sodium butyrate (Fig. 3B). The staining appeared heterogeneous: some cells were only basolaterally labeled, whereas other cells were fluorescent on both cell surface domains. Because random labeling seemed to coincide with more expression, we repeated the same experiment without induction with sodium butyrate. Indeed, primarily basolateral fluorescence was detected at low expression levels (Fig. 3C). Thus, the cytoplasmic domain of $\beta_1$ is also sufficient to mediate basolateral expression of the Fc receptor but seems to do so at a lower efficiency than that of $\alpha_5$. This is in agreement with the mixed cell surface polarity observed in cells co-expressing wild-type $\beta_1$ and $\alpha_5$CT9 (Fig. 1D).

The apical expression of the $\beta_1$ Fc receptor chimera at high expression levels could also have been due to a dominant negative effect of the integrin chimera causing a general defect in cell adhesion, as described for fibroblasts expressing a chimera containing the entire cytoplasmic domain of $\beta_1$ (29, 30, 31). We could not detect negative effects on the monolayer morphology, the polarized expression of another basolateral membrane protein (Na$^+/K^+$-ATPase), or on the localization of the tight junction protein ZO-1 in cells expressing FcR-$\beta_1$:11-47; such effects could easily be observed when a chimera containing the entire cytoplasmic domain of $\beta_1$ including the first 10 amino acids was expressed.

We next introduced different deletions into the cytoplasmic domains of the $\alpha_5$ and $\beta_1$ Fc receptor chimeras to map the regions in the cytoplasmic tails required for basolateral expression. We assayed the polarity of expression by binding the anti-Fc receptor antibody to either the apical or the basolateral cell surface domain of filter-grown transfected cells on ice. After washing, bound primary antibody was visualized with a secondary antibody conjugated to HRP and a colorimetric reaction. Fig. 3D shows that 80% of the chimera containing the entire cytoplasmic domain of $\alpha_5$ was detected basolaterally, confirming the confocal images shown above. In contrast, C-terminal deletions of 19 or 11 amino acids, respectively, resulted in preferentially apically expressed chimeras (FcR-$\alpha_5$:3-9 and FcR-$\alpha_5$:3-17). In contrast, removal of the membrane proximal domain, which is conserved in all $\alpha$ subunits (32), did not affect basolateral expression (FcR-$\alpha_5$:8-28). Thus, only the C-terminal part of the $\alpha_5$ cytoplasmic domain is required for basolateral expression of the chimeras.

We then expressed a set of $\beta_1$ Fc receptor chimeras and analyzed the polarity of expression without preincubation with sodium butyrate to avoid saturation. Fig. 3E shows that C-terminal truncation also resulted in apical expression (FcR-$\beta_1$:11-28 and FcR-$\beta_1$:11-38), indicating that the C-terminal part of the $\beta_1$ cytoplasmic domain is important for basolateral expression. Interestingly, substitution of tyrosine 44 by an alanine (FcR-$\beta_1$:11-47Y-A44) reduced the polarity of expression (~60% basolateral); hence, the tyrosine substitution resulted in only a partial inactivation of the basolateral sorting activity of the $\beta_1$ cytoplasmic domain because the complete absence of basolateral sorting information results in preferential apical expression of Fc receptors (28). Thus, the last nine amino acids of the
immunoprecipitated a basolateral plasma membrane domain. After extraction, the streptavidin-HRP (man cell surface, circumventing the apical plasma membrane do-

eral expression has thus far always been found to be achieved Surface Membranes—

- a integrin (and enhanced chemiluminescence to see the total biotiny-
tibody. Each precipitate was then split into three samples: (a)
tary labeled biotinylated integrin was visualized with HRP-streptavi-
(b) the apical cell surface insertion was monitored in at least two independent experiments.

- chase 0 min 15 min 30 min 60 min 120 min 16 hours

- A B A B A B A B A B A B A B A B A B A B

total IP

- surface 35S

- surface total

Fig. 4. Cell surface insertion of newly synthesized human αβ1 integrin and of chimeric constructs. Cell lines expressing either human αβ1 integrin (A and B), FcR-α5-38 (C), or FcR-β1-11-47 (D) were metabolically labeled for 20 min with [35S]methionine-methionine-cysteine and chased for the indicated periods of time, followed by selective biotinylation of either the apical (A) or basolateral (B) cell surface. After extraction, immunoprecipitations were performed using antibodies specific for α1 (A), β (B), or the Fc receptor (C and D). Each immunoprecipitate was then split into three samples of which one was directly analyzed by SDS-PAGE and fluorography to see total newly synthesized and immunoprecipitated protein (total IP), one was reprecipitated with streptavidin-agarose before electrophoresis and fluorography to detect newly synthesized protein at the cell surface (surface 35S), and the third was transferred to nitrocellulose after electrophoresis to visualize the steady-state distribution with streptavidin-HRP (surface total). Note that the same fraction of human β1 was found to be apically inserted when cells transfected with only the human β1 cDNA were analyzed, indicating that direct apical transport of β1 also occurred when it was associated with endogenous α subunits. For each type of transfection, cell surface transport was monitored in at least two independent experiments.

cytoplasmic domain of β1 are important for basolateral expres-
sion, and tyrosine 44 is involved in this process.

Newly Synthesized αβ1 Integrin Is Transported to Both Cell Surface Membranes—Cytoplasmic domain-mediated basolat-
eral expression has thus far always been found to be achieved by direct transport from the Golgi apparatus to the basolateral cell surface, circumventing the apical plasma membrane do-

- a integrin also mediate direct basolateral transport, we pulse-chase labeled transfected cells co-expressing wild-type aβ1 integrin and then bilitnated either the apical or the basolateral plasma membrane domain. After extraction, the human integrin was immunoprecipitated with the anti-α1 anti-

Each precipitate was then split into three samples: (a) the first sample was directly analyzed by SDS-PAGE and flu-
graphy to see the total labeled and immunoprecipitated integrin (i.e. newly synthesized integrin); (b) the second sample was reprecipitated with streptavidin-agarose to see the metabolic ly labeled biotinylated integrin (i.e. newly synthesized integrin at the cell surface); and (c) the third sample was fractionated by SDS-PAGE and transferred to nitrocellulose, and bilitnated integrin was visualized with HRP-streptavi-
din and enhanced chemiluminescence to see the total bilitnated integrin (i.e. the steady-state cell surface distribution of human αβ1 integrin).

Fig. 4A shows that α5 was synthesized as a higher molecular weight precursor (heaviest band at 0 min) and then processed and also started to co-immunoprecipitate β subunits. The total immunoprecipitated α5 appeared to increase with time, sug-

gesting that the monoclonal antibody used in this experiment recognizes the mature forms more efficiently than the early forms, a common behavior of monoclonal antibodies that is often used to assay conformational maturation of proteins in the early secretory pathway (for examples, see Refs. 33 and 34). Newly synthesized integrin was detected on the cell surface after 60 min of chase (Fig. 4A, surface 35S proteins) and started to appear at both cell surface domains in similar amounts. At longer times of chase, the integrin started to disappear from the apical cell surface, whereas it remained at the basolateral domain. Total biotinylated integrin exhibited a clear basolat-
eral expression in all cultures, suggesting that the apical appear-

- aβ1 Fc receptor chimera appeared preferentially at the apical cell surface but were detected basolaterally if the steady-state distribution was analyzed (Fig. 4, C and D). Thus, newly synthesized human α5β1 integrin and the chimeras are transported to both cell surface domains.

aβ1 Integrin Is More Stable at the Basolateral Cell Surface than at the Apical Cell Surface—Basolateral polarization of the transfected integrin could be achieved by internalization from the apical membrane followed by either transcytosis to the basolateral cell surface or degradation. We found that the cytoplasmic domains of α5 and β1 were both able to mediate efficient internalization of Fc receptor chimeras, a prerequisite for both mechanisms (data not shown). Because we observed that large fractions of newly synthesized α5β1 integrin and chimeras were degraded relatively quickly, we tested whether apical and basolateral α5β1 integrins have different half-lives. To do this, we biotinylated filter-grown cells either apically or basolaterally and then incubated the cells for different periods of time at 37 °C. The cells were then lysed, and the integrin was immunoprecipitated basolateral integrin. If the immunoprecipitation was done with anti-β1 antibodies, the apical cell surface appear-

ance was even more pronounced (Fig. 4B). It is currently not clear what caused the difference in the amounts of apically detected integrin in Fig. 4A and B, but it could be that a fraction of α5 formed dimers with a relatively short half-life (and therefore did not influence the steady-state distribution) with an endogenous β subunit that, in contrast to β1, is able to mediate direct basolateral transport. Moreover, preferential apical cell surface insertion was also obtained when the human β1 subunit was expressed alone and thus could only dimerize with endogenous α subunits (data not shown), excluding that apical transport was induced by human α5. Because the monoclonal anti-β1 antibodies that were at our disposition did not immunoprecipitate canine β1, we could not directly test whether endogenous β1 is also transported to the apical membrane. Nevertheless, endogenous β1 integrins have previously been reported on the apical cell surface of MDCK cells, excluding that the apical expression is a transfection artifact due to, for instance, higher expression levels (18–20). Because the apical integrin pool has a short half-life (see below), the differ-
cent polarity of cell surface insertion observed by different in-
vestigators might be due to the different times of chase used (35, 20). In agreement with the results obtained with the di-

meric integrin, the α5 and the β1 Fc receptor chimera appeared preferentially at the apical cell surface but were detected basolaterally if the steady-state distribution was analyzed (Fig. 4, C and D). Thus, newly synthesized human α5β1 integrin and the chimeras are transported to both cell surface domains.

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ance was not due to preferential apical biotinylation or an inability of the antibody (or of the technique used) to immuno-

- aβ1 integrin and then biotinylated either the apical or the basolateral plasma membrane domain. After extraction, the human integrin was immunoprecipitated with the anti-α1 anti-

Each precipitate was then split into three samples: (a) the first sample was directly analyzed by SDS-PAGE and flu-
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- chase 0 min 15 min 30 min 60 min 120 min 16 hours

- A B A B A B A B A B A B A B A B A B A B

total IP

- surface 35S

- surface total
immunoprecipitated with either anti-α or anti-β antibodies. The immunoprecipitates were fractionated by SDS-PAGE and transferred to nitrocellulose, and biotinylated integrin was detected with streptavidin-HRP.

Fig. 5 shows the result of such an experiment in which samples from the same cell extracts were immunoprecipitated with either anti-α (A) or anti-β (B) antibodies. In both cases, apically biotinylated integrin disappeared relatively quickly ($t_{1/2} \sim 1.5$ h), whereas basolaterally biotinylated integrin was very stable and became only slowly degraded ($t_{1/2} \geq 16$ h). Thus, αβ integrin is more stable at the basolateral cell surface than at the apical cell surface. To test whether the cytoplasmic domains of the α and β subunits are sufficient to mediate selective stabilization in the basolateral membrane, we repeated the same experiment with MDCK cells expressing either one of the two integrin/Fc receptor chimeras. Fig. 6 shows that both the α (A) and the β (B) chimera exhibited a similar behavior as the integrin; apically biotinylated chimeras were more quickly degraded ($t_{1/2} < 60$ min) than basolaterally labeled chimeras ($t_{1/2} \geq 180$ min). Although basolaterally expressed chimeras were also more stable than apical ones, they were not as stable as basolateral αβ integrin. Because apical chimeras and apical integrins exhibited comparable half-lives, this did not seem to be due to quicker proteolysis of internalized chimeras but to more efficient stabilization of the basolateral pool of dimeric integrin, due to cooperation between the two cytosolic domains and/or interactions occurring in the extracellular matrix. Nevertheless, the cytoplasmic domains of α and β were independently sufficient to mediate stabilization of basolaterally expressed chimeras. Although these experiments do not completely exclude that small fractions of apical chimeras and apical αβ integrin are transcytosed, the degradation of apically expressed integrin is too fast for efficient transcytosis to occur and too complete for transcytosis to be responsible for a significant amount of the finally basolaterally expressed protein (i.e., the apically biotinylated integrins do not exist anymore). Thus, basolateral polarization of wild-type and chimeric integrins is achieved by selective stabilization of the basolaterally expressed pool.

**Fig. 5.** Stabilization of human αβ integrin on the basolateral cell surface. Filter-grown and sodium butyrate-treated MDCK cells co-expressing αβ were apically or basolaterally biotinylated on ice. After incubation at 37°C for the indicated periods of time, the cells were extracted, and immunoprecipitations were performed with either anti-α (A) or anti-β (B) antibodies. After fractionation by SDS-PAGE and transfer to nitrocellulose of the immunoprecipitates, the presence of biotinylated integrin was visualized with streptavidin-HRP and chemiluminescence. The films were then quantified by densitometric scanning, and the signals obtained after no incubation at 37°C were set to 100%.

**Fig. 6.** Basolateral stabilization of chimeric Fc receptors containing the cytoplasmic domain of α or β. Cells expressing either FeR-αα, 3-28 (A) or FeR-β, 11-47 (B) were biotinylated and incubated at 37°C as described in the Fig. 5 legend. The chimeras were then immunoprecipitated with the anti-Fc receptor antibody, and biotinylated immunoprecipitated protein was again visualized after SDS-PAGE and blotting to nitrocellulose with streptavidin-HRP and chemiluminescence. The films were quantified as described in the Fig. 5 legend. Note that when the cells were radioactively labeled before the experiment, and total immunoprecipitated chimera was directly detected by SDS-PAGE and fluorography, disappearance was paralleled by the appearance of a smear of immunoprecipitated protein indicating the protein was degraded and not sheared into the medium (data not shown).

**DISCUSSION**

Our experiments indicate that basolateral polarization of transfected human αβ integrin in MDCK cells is mediated by distinct determinants in the cytoplasmic domains of each subunit, similar to other basolateral membrane proteins. In contrast to the basolateral sorting determinants described thus far, the cytoplasmic domains of α and β make use of an alternative sorting mechanism that does not involve direct basolateral sorting in the Golgi complex but involves selective stabilization at the basolateral cell surface after transport to both cell surface domains.

Newly synthesized αβ integrin and chimeras containing the cytoplasmic domain of either one of the two subunits were transported to both cell surface domains. This was also observed when MDCK cells were only transfected with β cDNA, indicating that the apical cell surface transport was not due to the α subunit, which is normally not an abundant subunit in MDCK cells (17), but also occurred when the human β subunit was associated with endogenous α subunits. Because the existence of endogenous β integrins in the apical membrane of MDCK cells has been reported previously, apical transport of transfected human β is apparently not just caused by a species difference between human and dog β (18–20).

Basolateral polarization aided by selective stabilization has also been observed for Na+K+-ATPase (11, 14). In this case, significant apical transport of newly synthesized protein was only observed in MDCK cells deficient in apical glycolipid sorting (13, 14). Importantly, our MDCK strain II cells efficiently sort many apical and basolateral membrane proteins including Na+K+-ATPase and apically expressed glycocephalysphatidylinositol-anchored proteins and are hence able to recognize all known types of plasma membrane sorting signals ranging from cytoplasmic basolateral targeting determinants over glycosphatidylinositol anchors to extracytoplasmic apically sorting carbohydrates (5, 10, 21, 25, 28, 36).

Although the α and the β cytoplasmic domains were independently sufficient to mediate basolateral polarization, basolateral stabilization of the wild-type integrin was more pronounced than stabilization of the chimeras. One possible
reason for this is that two cytoplasmic domains may be better than one because at least two interactions, perhaps occurring in a cooperative manner, can be used for stabilization. It could also be that the transmembrane and extracytoplasmic domains of the wild-type integrin participate in basolateral stabilization (e.g. substrate binding). Nevertheless, the chimeras clearly indicate that the cytoplasmic domain sequences that were required for basolateral polarization of the dimeric integrin were also sufficient to mediate basolateral polarization of the truncated Fc receptor, which has been shown to be expressed only basolaterally if its cytoplasmic domain contains sorting signals specific for the basolateral membrane (28, 21, 37, 38).

Stabilization of the basolateral pool of integrins could occur either by selective recycling of basolaterally internalized molecules or by interactions with the submembrane cytoskeleton at the cell surface. Cytoplasmic determinants that mediate basolateral recycling are similar or even identical to determinants that mediate basolateral sorting in the Golgi apparatus (25, 39); hence, it is unlikely that the $\alpha\beta_1$ integrin possesses a determinant for basolateral recycling, because it is not efficiently sorted to the basolateral membrane during biosynthesis or after internalization from the apical membrane. Nevertheless, the short half-lives of apically expressed chimeras and integrin made it impossible to measure differences in endocytic trafficking. Because basolaterally recycling receptors like those for low density lipoprotein or transferrin exhibit extensive endosomal localization in the steady state, the absence of clear endosomal staining for integrins in our immunofluorescence experiments suggests that stabilization occurs at the basolateral cell surface. Although we were not able to detect significant Triton X-100 insolubility of basolaterally expressed constructs (data not shown), it is likely that basolateral stabilization of the $\alpha\beta_1$ integrin is mediated by interactions with cytoskeletal components. Integrins are connected to the actin-based cytoskeleton by a linkage consisting of a large complex of proteins that is also involved in the transmission of signals across the plasma membrane. In fibroblasts, integrins, which normally become quickly degraded, have been shown to become stabilized on the plasma membrane upon the addition of ligand (40). Importantly, the addition of ligand stabilizes not only the interacting integrin but also other integrins that cannot interact with the added ligand (40), indicating that the stabilization of integrins at the plasma membrane can also be mediated by their cytoplasmic domains in fibroblasts. Thus, in the steady state, cytoplasmic domain-mediated stabilization in epithelial cells occurs at the basolateral plasma membrane domain because it contains established extracellular matrix contacts and therefore the necessary organization of the submembrane cytoskeleton.

Of all the proteins known to interact with integrin cytoplasmic domains, only calreticulin is known to bind to $\alpha$ subunits by interacting with a membrane-proximal conserved sequence (41); this conserved sequence was neither sufficient for basolateral expression of the dimeric integrin nor required for basolateral expression of the $\alpha_5$-Fc receptor chimera. In contrast, the last 11 amino acids of the $\alpha_5$ subunit were found to be required for basolateral expression. Although this could be due at least in part to reduced rates of endocytosis, the C-terminal 11 amino acids do not contain a motif reminiscent of an endocytosis determinant, suggesting a direct involvement of the C-terminal 11 amino acids in basolateral stabilization.

The cytoplasmic domain of $\beta_1$ is known to interact with many different proteins, and the binding sites cover essentially the entire cytoplasmic domain (15, 41, 42). Similar to $\alpha_5$, the C-terminal amino acids of $\beta_1$ were required for basolateral expression. Although the last 9 amino acids contain a NPXY motif, of which the tyrosine is apparently involved in basolateral expression, previous studies indicate that the C-terminal NPXY motif is not important for integrin internalization but for the accumulation of $\alpha\beta_1$ in focal adhesions (43–46). Of all the proteins known to interact with the cytoplasmic domain of $\beta_1$, only one of the proposed binding sites for talin and one for focal adhesion kinase overlap with the C-terminal domain required for basolateral expression (47). Additionally, the tyrosine residue involved in basolateral expression is also important for the interaction with the recently identified protein ICAP-1 (48).
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