

Transport of the IgE Receptor α -Chain Is Controlled by a Multicomponent Intracellular Retention Signal*

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The human high affinity IgE receptor (Fc ϵ RI) is a central component of the allergic response and is expressed as either a trimeric $\alpha\gamma 2$ or tetrameric $\alpha\beta\gamma 2$ complex. It has been previously described that the cytoplasmic domain (CD) of the α -chain carries a dilysine motif at positions $-3/-7$ from the C terminus that functions in intracellular retention prior to assembly with other Fc ϵ RI subunits. In this report we have further explored the role of the $-3/-7$ dilysine signal in controlling steady-state α -chain transport by mutational analysis and found little surface expression of a $-3/-7$ dialanine α -chain mutant but significant Golgi localization. We compared the transport properties of a series of α -chain cytoplasmic domain truncation mutants and observed that truncation mutants lacking 23 or more C-terminal residues showed a dramatic increase in steady-state transport suggesting a role for the membrane-proximal CD sequence in α -chain retention. By performing alanine-scanning mutagenesis we identified a dilysine sequence (Lys²¹²–Lys²¹⁶) proximal to the transmembrane domain (TMD) that is important for both α -chain cell-surface expression and intracellular stability. Furthermore, co-mutation of the Lys²¹²–Lys²¹⁶ residues with the $-3/-7$ dilysine signal produced a dramatic increase in α -chain surface expression that was further increased by co-mutation of the lone charged residue (Asp¹⁹²) in the TMD thereby defining three regions that function to regulate α -chain transport and in a highly synergistic manner.

The high affinity IgE receptor (Fc ϵ RI)² is a multisubunit complex comprised of either a tetrameric $\alpha\beta\gamma 2$ complex or an alternative trimeric $\alpha\gamma 2$ isoform (1, 2). Aggregation of the tetrameric receptor on mast cells and basophils occurs upon cross-linking of receptor-bound IgE with a multivalent antigen that initiates Fc ϵ RI-dependent signaling, culminating in cellular degranulation and the ensuing clinical symptoms of hypersensitivity. Expression of the trimeric Fc ϵ RI isoform occurs on a number of cells, including monocytes, epidermal Langerhans cells, and dendritic cells, and a role for the $\alpha\gamma 2$ receptor in antigen presentation has now been established (3). The Fc ϵ RI α -chain is exclusively involved in IgE binding, whereas the associated γ - and β -chains function in sig-

naling by the presence of intracellular tyrosine activation motifs. Assembly with the β -chain also functions to amplify Fc ϵ RI cell-surface expression relative to the $\alpha\gamma 2$ isoform (4, 5) and represents a fundamental mechanism for the regulation of Fc ϵ RI cell-surface expression. It has long been known that binding of IgE to cell-surface Fc ϵ RI leads to enhanced receptor expression (6), an effect that has now been amply demonstrated for both receptor isoforms (6–9) and is thought to involve receptor-ligand surface stabilization and possibly increased export of α -chain from an intracellular pool (10, 11). Additional mechanisms that regulate Fc ϵ RI expression have also been identified, including the effects of interleukin-4 in mast cells (7, 12) and transforming growth factor- $\beta 1$ in both dendritic cells (13) and mast cells (14). Because the sensitivity of the allergic response is critically linked to Fc ϵ RI surface expression (15) that in turn is dependent on Fc ϵ RI subunit transport characteristics (16) and assembly (17), it remains an important goal to fully elucidate the Fc ϵ RI structural features that regulate these processes to thereby provide a better understanding of the molecular origins of the allergic response.

The underlying factors that control Fc ϵ RI export from the ER to the plasma membrane are important in understanding regulation of Fc ϵ RI surface expression. The minimum post-translational requirements for Fc ϵ RI α -chain cell-surface localization include ER-derived N-linked core glycosylation (16, 18, 19) and intracellular assembly with the homodimeric Fc ϵ RI γ -subunit (17). In transfection studies performed in the absence of γ -chain, the α -chain has been shown to achieve core glycosylation and is retained and accumulates in the ER in a form that binds IgE (16). A recent study has shown that the co-translational assembly of multimeric Fc ϵ RI in the ER is an important step in regulation of Fc ϵ RI surface expression (17). Several reports have now appeared describing the intracellular accumulation of α -chain in certain cell types. For example α -chain has been detected in Fc ϵ R γ -positive eosinophils without detectable Fc ϵ RI surface expression (20, 21), although a secreted form of a truncated α -chain was found (20). Megakaryocytes have also been reported to express only an intracellular localized α -chain (22), and interleukin-4 has been shown to induce an intracellular accumulation of α -chain in *in vitro* generated dendritic cells (23). In addition it has also been demonstrated that $\alpha\gamma 2$ receptor complexes show significantly higher intracellular accumulation than the corresponding tetrameric receptor isoform (17), suggesting the possibility that the factors responsible for intracellular retention of the Fc ϵ RI α -chain may also function in partial retention of the trimeric Fc ϵ RI complex.

It has been previously shown using chimeric reporter molecules composed of the interleukin-2 receptor α -chain (CD25 and Tac) and C-terminal Fc ϵ RI α cytoplasmic domain (CD) sequences (Tac- α) that the two lysine residues near the C terminus (positions -3 and -7) function in ER retention during early biosynthesis (18). In that work it was suggested that after assembly with the Fc ϵ RI γ -chain the $\alpha\gamma 2$ complex becomes transport competent by a mechanism involving steric masking of the dilysine signal by proximal residues of the γ -chain CD. Numerous

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² The abbreviations used are: Fc ϵ RI, high affinity receptor for IgE; BFA, brefeldin A; CD, cytoplasmic domain; TMD, transmembrane domain; COPI, coatamer complex I; ERGIC, endoplasmic reticulum Golgi intermediate compartment; Tac, interleukin-2 receptor α -chain (CD25); MFI, mean fluorescence intensity; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; Ab, antibody; mAb, monoclonal antibody; wt, wild type; FACS, fluorescence-activated cell sorting; endo H, endoglycosidase H.

TABLE 1

Sequence of PCR oligonucleotides used in this study

Oligonucleotide ^a	Construct name	Oligonucleotide sequence (5' → 3')
1	Tac ^b	GACTGGATCCGTCAGGAAGATGGATTACATCTG
2	Tac	GATCCTCGAGCTAGATTGTTCTTCTACTCTTCC
3	Tac-PKPNPKNN ^c	GATCCTCGAGCTAGTTGTTCTTTGGGTTTGGCTTAGGGATTGTTCTTCTACTCTTCC
4	Tac-PAPNPANN	GATCCTCGAGCTAGTTGTTCTGCTGGGTTTGGCGCAGGGATTGTTCTTCTACTCTTCC
5	Tac-PAPNPKN	GATCCTCGAGCTAGTTGTTCTTTGGGTTTGGAGCAGGGATTGTTCTTCTACTCTTCC
6	Tac-A4KAAA	GATCCTCGAGCTAAGCTGCCTTTTTTCGACGCGGCAGCGATTGTTCTTCTACTCTTCC
7	Tac-A3K3AA	GATCCTCGAGCTAAGCTGCCTTTTTCTAGCGGCAGCGATTGTTCTTCTACTCTTCC
8	Tac-A3KAKAA	GATCCTCGAGCTAAGCTGCCTTTCGCTTAGCGGCAGCGATTGTTCTTCTACTCTTCC
9	Tac-AKA3KAA	GATCCTCGAGCTATGCGGCTTTAGCCGCTGCCTTGGCGATTGTTCTTCTACTCTTCC
10	Tac-A5KAA	GATCCTCGAGCTAAGCTGCCTTTGCCGAGCGGCAGCGATTGTTCTTCTACTCTTCC
11	Tac-A8	GATCCTCGAGCTAAGCTGCCTTTCGCGCAGCGGCAGCGATTGTTCTTCTACTCTTCC
12	Tac-αCD ^d	GTGACCTGCTGCTGAGTTGAGAGCCCACTCAGGAGGAGGAC
13	Tac-αCD ^{e,f}	TCAACTCAGCAGCAGGTCAC
14	huFceRIα ^g	GACTGGATCCGAGTCCATGAAGAAGATGGCTCCTGCC
15	huFceRIα	GACTCTCGAGTCAACCAATGTTAATTGAGCACTTACT
16	αK226A/K230A	CTCGAGTCAGTTGTTTGGCGGGTTTGGCGCAGGATGTGGGTTTCAAGATCTGAAG
17	α-CD2 ^h	GACTCTCGAGTTAAGTTGAGATAAATAATCCTGTGTCCACAGC
18	α-CD10	GACTCTCGAGTTACAAGAGAAATGTGACCTGCTGCTGAG
19	α-CD22	GACTCTCGAGTTACAGAAGTCTGAAGCCTTCTGTTCTCTT
20	α-CD17	GACTCTCGAGTTATTTCTGGTTCTCTTAATCTTC
21	αK210A	GGGTTTCAAGAAGTCTGAAGCCTTCTGTTCTCTTAATCGCCAAGAGAAATGTGACCTGCTG
22	αK210A ⁱ	GACTCTCGAGTCAGTTGTTTGGGTTTGGCTTAGGATGTGGGTTTCAAGATCTGAAGCC
23	αK212A ^j	GGGTTTCAAGAAGTCTGAAGCCTTCTGTTCTCGCAATCTTCAAGAGAAATGTGACCTGCTG
24	αK212A/K216A ^k	GTGGGTTTCAAGAAGTCTGAAGCCTGCTGTTCTGCAATCTTCAAGAGAAATG
25	αK212A/R213A/ R215A/K216A ^l	GTGGGTTTCAAGAAGTCTGAAGCCTGCCGCGTCCGCGCAATCTTCAAGAGAAATG
26	αK4A4 ^m	GACTTCTGAACCCACATCCTGCGCCAAACCCAGCAAACTGACTCGAGAGTC
27	αD192A	GGAGATGAACAAGCCCGTGGCCACAGCAAAACAAGATCACC
28	αD192A ⁿ	CACGGGCTTGTTTCATCTCCACTCAGCAGCAGGTCACATTTT

^a Unless indicated otherwise all oligonucleotides anneal to the template cDNA (–) strand.^b Anneals to the 5'-end of the Tac cDNA (+) strand.^c All Tac-α chimeras were produced using oligonucleotide 1 as the forward PCR primer.^d Tac cDNA was used as the PCR template.^e PCR with primer 15 using hu FceRI α-chain cDNA as template.^f Tac-αCD was produced using the PCR product from oligonucleotides 1 and 12 plus the PCR product from oligonucleotides 13 and 15 as template in a second stage PCR using primers 1 and 15. Tac-αCD-K226A/K230A, Tac-αCD-K212A/K216A, and Tac-αCDK4A4 were prepared by a similar strategy.^g All α-chain constructs were prepared using oligonucleotide 14 as the forward PCR primer.^h Oligonucleotide 17 was also used to produce αCD2/D192A from D192A template DNA.ⁱ αK210A was produced using the PCR product from oligonucleotides 14 and 21 as template.^j αK212A was produced using the PCR product from oligonucleotides 14 and 23 as template in a second stage PCR using oligonucleotides 14 and 22.^k αK212A/K216A was produced using the PCR product from oligonucleotides 24 and 14 as template in a second stage PCR using oligonucleotides 22 and 14.^l αK212/R213A/R215A/αK216A was produced using the PCR product using oligonucleotides 25 and 14 as template in a second PCR using oligonucleotides 22 and 14.^m αK212A/K216A was used as PCR template.ⁿ αD192A was produced using the PCR products using oligonucleotides 14 and 27 plus 15 and 28 in a second stage PCR using primers 14 and 15. αD192A/K226A/K230A, αD192A/K212A/K216A, and αD192A/K4A4 were prepared by a similar strategy.

secretory proteins contain ER targeting signals such as the C-terminal sequences KDEL/HDEL (24) and the canonical dilysine motifs KKXX- and KXKXX-COOH (25). Dilysine signals target ER localization by retrieval from the Golgi apparatus (25) by interaction with the multimeric coat protein 1 complex (COPI (26–28)). ER retention/retrieval motifs are found in many ER resident proteins (24, 29, 30) as well as in subunits of various oligomeric membrane receptors such as the T cell receptor-CD3 complex (31–34), the nicotinic acetylcholine receptor (35), and the ε-aminobutyric acid receptor (36), among others. Additional ER localization signal sequences have also been identified, including diarginine-based (RXR) CD sequences (36, 37) as well as certain TMD sequences (38, 39).

In this report we explored the function of the FceRIα –3/–7 dilysine signal using Tac reporter molecules and established that the –3/–7 dilysine signal is only partially functional in controlling steady-state cell-surface expression and is less efficient in ER retention/retrieval than canonical KKXX-COOH dilysine signals. We also observed that expression of a –3/–7 dialanine α-chain mutant showed little capacity for surface expression, leading us to postulate that additional α-chain sequence might be involved in intracellular retention. By using a series of α-chain truncations and site-directed mutations, we have identified a short polybasic sequence (Lys²¹²-Arg-Thr-Arg-Lys²¹⁶) in the middle of the primary CD sequence that functions in intracellular retention. In addition, the single charged residue (Asp¹⁹²) in the α-chain TMD was

also found to contribute significantly to α-subunit transport. Thus the FceRI α-chain contains a three-component ER retention signal that functions to stringently retain the unassembled α-chain in the ER prior to assembly with other FceRI subunits.

MATERIALS AND METHODS

Expression Constructs—The construction of human FceRI α- and γ-chain expression plasmids has been previously described (16). All additional constructs used in this study were produced by PCR amplification of coding sequences using *Pfu* turbo polymerase (Stratagene) and the forward and reverse oligonucleotide primers (high-performance liquid chromatography-purified, Integrated DNA Technologies, Coralville, IA) summarized in Table 1 followed by cloning into pcDNA 3.1 (Invitrogen). Typical PCR amplification conditions used in this study included an initial 2-min denaturation step at 94 °C and then 25 cycles of denaturation (94 °C), annealing (55–60 °C), and extension (72 °C) followed by a final 10-min 72 °C incubation step. Full-length (830 bp) Tac coding cDNA was produced by reverse transcription-PCR from mRNA isolated from Jurkat cells cultured in RPMI medium supplemented with 5 μg/ml Phytohemagglutinin-M (Invitrogen) as described (40). PCR products were produced with BamHI and XhoI cloning sites, and the sequence of all clones was confirmed using an ABI PRISM 3100 sequencer.

Cell Lines and Transfections—HEK293 and COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Irvine Scientific, Irvine, CA), 2 mM glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. For transfection, HEK293 cells were seeded at 9.5×10^5 cells/well in a 6-well plate and transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol and harvested 40 h later by trypsinization. For confocal microscopy, COS-7 cells were seeded at 5.0×10^5 cells/well and then transfected as above using 2 μ g of plasmid DNA per well. After 24 h, cells were harvested, resuspended in DMEM containing 10% fetal bovine serum, and then seeded in poly-D-lysine pre-coated eight-chambered Lab-Tec II coverglass (Nalge Nunc, Naperville, IL) and cultured for an additional 24 h prior to antibody staining as described below.

Flow Cytometry—Transfected cells were stained with either anti-Fc ϵ RI α -chain mAb 15/1 (41) or isotype control mAb MOPC21 (mIgG₁/k, Sigma, 0.8 μ g/ 10^6 cells), washed, and then sequentially treated with biotinylated goat F(ab')₂ anti-mouse IgG₁ (0.5 μ g/ 10^6 cells, Southern Biotechnology Associates, Birmingham, AL) and streptavidin-phycoerythrin (0.5 μ g/ 10^6 cells, BD Pharmingen). Labeled cells were then resuspended in 0.5% bovine serum albumin in phosphate-buffered saline containing 1 μ g/ml propidium iodide (Sigma) and analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest Pro Software for both acquisition and analysis. Live cells were gated by propidium iodide exclusion using the FL3 channel.

Pulse-chase Analysis—COS-7 transfectants were washed once with methionine-free DMEM containing 5% dialyzed fetal bovine serum and then allowed to incubate in the same medium for 15 min prior to labeling with 250 μ Ci/ml [³⁵S]methionine for 15 min at 37 °C. The labeling medium was then replaced with DMEM containing 10% fetal bovine serum for different chase intervals. For each chase time cells were collected, washed, and pelleted by centrifugation, and lysates were prepared for subsequent immunoprecipitation as described below.

Immunoprecipitation, Endoglycosidase H Treatment, and Western Blot Analysis—Cells were solubilized in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA) containing protease inhibitors (Complete Protease Inhibitors™, Sigma) for 30 min on ice and then centrifuged at $13,000 \times g$ to remove insoluble debris. Lysate supernatants were precleared with protein G-Sepharose beads (Amersham Biosciences) and then treated with 5 μ g of anti-CD25 mAb (clone 7G7/B6, Upstate Biotechnology, Lake Placid, NY) pre-bound to protein G-Sepharose beads for 16 h at 4 °C. Resin-bound protein was eluted with SDS-PAGE sample buffer. In pulse-chase experiments gels were dried and analyzed by autoradiography. Other samples were treated with or without 10^4 units of Endoglycosidase H (New England Biolabs, Beverly, MA) for 16 h following the manufacturer's protocol. Proteins were then fractionated by SDS-PAGE (4–20% gradient), transferred to Immobilon-P membrane (Millipore, Bedford, MA), and probed with a rabbit polyclonal anti-CD25 (sc-666, Santa Cruz Biotechnology, Santa Cruz, CA) followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad). The same blots were also probed with an anti- γ -COPI Ab (sc-14167) or an anti- β -COPI Ab (sc-13335, both antibodies from Santa Cruz Biotechnology). For Fc ϵ RI α -chain immunoblots, a rabbit polyclonal anti-Fc ϵ RI α -chain Ab (Upstate Biotechnology) was used in combination with a horseradish peroxidase-conjugated goat anti-rabbit IgG. The horseradish peroxidase conjugates were visualized using ECL Western blotting detection reagents (Amersham Biosciences).

Neomycin Precipitation of COPI Complexes—COPI complexes were precipitated from transfectant lysates essentially as previously described (42, 43). Briefly, HEK293 transfectants were suspended in lysis buffer (0.1% Triton X-100, 25 mM HEPES, 50 mM KCl, 2.5 mM Mg(OAc)₂, pH

7.4) and solubilized by cavitation using a 25-gauge syringe needle. After centrifugation, 2-fold serial dilutions of lysate were prepared in lysis buffer without Triton X-100 and centrifuged at $15,000 \times g$ for 30 min at 4 °C. The protein concentration was determined for each dilution using the DC Protein assay kit (Bio-Rad). Thereafter neomycin sulfate (Sigma) was added to a final concentration of 1 mM, and the mixture was incubated for 2 h at 4 °C. The precipitate was collected, washed, and then treated with SDS-PAGE sample buffer and processed as described above.

Confocal Microscopy—Transfected cells were washed once with DMEM, fixed with ice-cold 4% paraformaldehyde, washed twice with phosphate-buffered saline, and then incubated for 10 min with 0.1% Triton X-100 in phosphate-buffered saline. Nonspecific binding was blocked by incubation with 5% whole goat serum (Sigma) in phosphate-buffered saline containing 0.1% Triton X-100. Cells were then washed and allowed to incubate for 90 min at ambient temperature with 20 μ g/ml mAb 15/1 together with either an anti-calnexin Ab (SPA-860, Stressgen, Victoria, Canada) or anti-giantin Ab (Covance, Denver, PA). To visualize co-localization within the ERGIC, an anti-ERGIC-53 mAb (generously provided by Prof. H. Hauri, University of Basel) was used together with a polyclonal rabbit anti-Fc ϵ RI α Ab (Upstate). Cells were then washed and treated with either FITC-labeled secondary Ab (FITC-goat anti-mouse IgG₁, Southern Biotech Associates), Texas Red-labeled goat anti-rabbit IgG (Southern Biotech Associates), Cy5-labeled goat anti-rabbit IgG (Zymed Laboratories Inc., San Francisco, CA), or Cy5-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Secondary reagents were diluted 1:100 in blocking solution and incubated with cells for 1 h at ambient temperature, washed, and then treated with SlowFade Light Antifade mounting medium (Molecular Probes-Invitrogen, Eugene, OR) according to the manufacturer's instructions. An MRC1024 laser scanning confocal microscope (Bio-Rad), equipped with a krypton/argon mixed gas laser with excitation wavelengths of 488, 568, and 647 nm and attached to a Zeiss Axiovert S100TV inverted microscope with Infinity Corrected Optics and a C-Apo 40 $\times$ objective, was used to analyze labeled cells. Images were collected on the confocal microscope using LaserSharp (version 3.2) software (Bio-Rad) and processed with Photoshop software (Adobe, San Jose, CA).

RESULTS

Role of Fc ϵ RI α -Chain –3/–7 Lysine Residues in Controlling Steady-state Surface Expression—Although intracellular retention of the unassembled Fc ϵ RI α -chain is generally attributed to the function of a C-terminal dilysine signal (18), the precise capacity of this motif and the potential role of other Fc ϵ RI α -chain CD sequence in controlling α -chain transport has not been determined. To test the functional capacity of the –3/–7 dilysine signal to control ER export, we employed Tac reporter molecules expressing the C-terminal eight residues of the Fc ϵ RI α -chain (Tac-PKPNPKNN, referred hereafter as Tac-KK) and the –3/–7 dialanine mutant Tac-PAPNPANN (designated Tac-AA) and compared the capacity of each to accumulate on the surface of transfected HEK293 cells. We first examined the kinetics of surface expression of the two Tac- α reporter molecules compared with wild type (wt) Tac over intervals ranging from 10 to 40 h post-transfection and found that surface expression of wt Tac and Tac-AA were uniformly similar over time, whereas Tac-KK expression was significantly lower at each post-transfection time (shown for $t = 10$ h in Fig. 1A). The possibility that differential surface expression was simply due to differences in protein expression was assessed by comparison of whole cell lysate Western blots. As shown in the Fig. 1A immunoblot, no significant

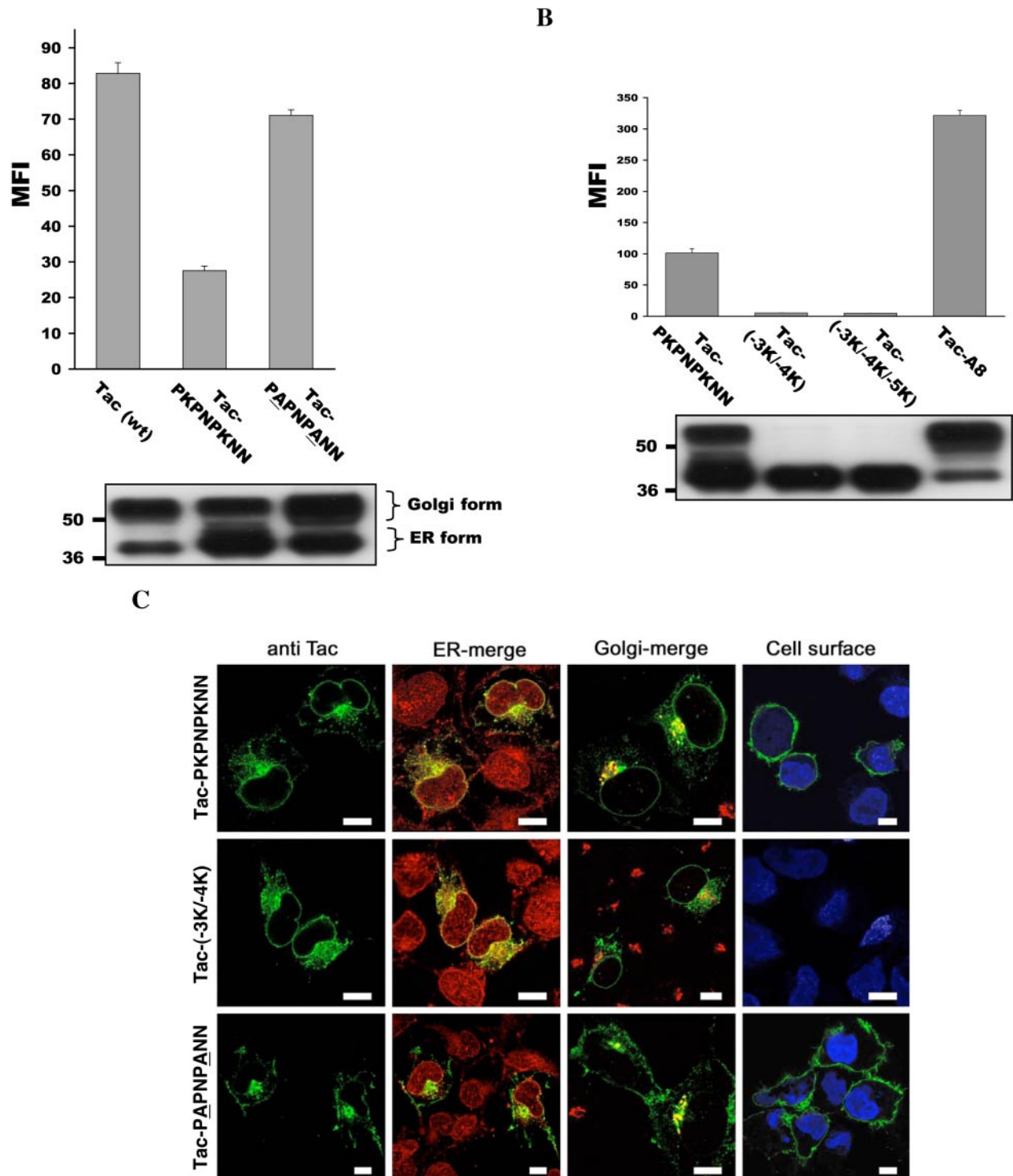


FIGURE 1. Steady-state expression of Tac- α chimeric reporter molecules in transiently transfected HEK293 cells. *A*, the cell-surface expression profile of wt Tac, Tac-PKPNPKNN, and Tac-PAPNPANN determined 10 h after transfection of equivalent DNA amounts was determined by FACS analysis. The average MFI values $\pm$ S.E. from quadruplicate transfections are shown. The corresponding total protein expression for each transfectant was determined by whole cell lysate immunoblotting. The ER- and Golgi-processed forms of each transfectant and the position of molecular mass markers (in kilodaltons) are indicated. *B*, summary of steady-state surface expression and total Tac-related protein expression of Tac-PKPNPKNN, Tac-(-3K/-4K), (Tac-3K/-4K/-5K), and Tac-A8 in transiently transfected HEK293 cells. The bar graph summarizes surface expression levels as average MFI values $\pm$ S.E. from quadruplicate transfections, and the total Tac-related protein expression is summarized in the accompanying immunoblot of whole cell extracts from an equivalent number of transfected cells. *C*, comparison of Tac-PKPNPKNN, Tac-(-3K/-4K), and Tac-PAPNPANN sub-cellular localization by confocal microscopy in transfected COS-7 cells. The ER-merge shows simultaneous staining with anti-Tac and anti-calnexin Abs, and the Golgi-merge shows simultaneous staining with anti-Tac and anti-giantin Abs. The far right column shows anti-Tac cell-surface staining in non-permeabilized cells that were also incubated with TO-PRO iodide for visualization of the nucleus. White scale bars represent 10 μ m.

differences in total protein expression (Golgi plus ER forms) between the two constructs was observed, although relatively more of the Tac-AA Golgi form was detected. To further define the functional

capacity of the -3/-7 dilysine signal in ER retention/retrieval, we compared the expression of Tac-KK to Tac reporters showing either optimal ER retention (Tac-AAAAKKAA, designated Tac-(-3K/-4K), and

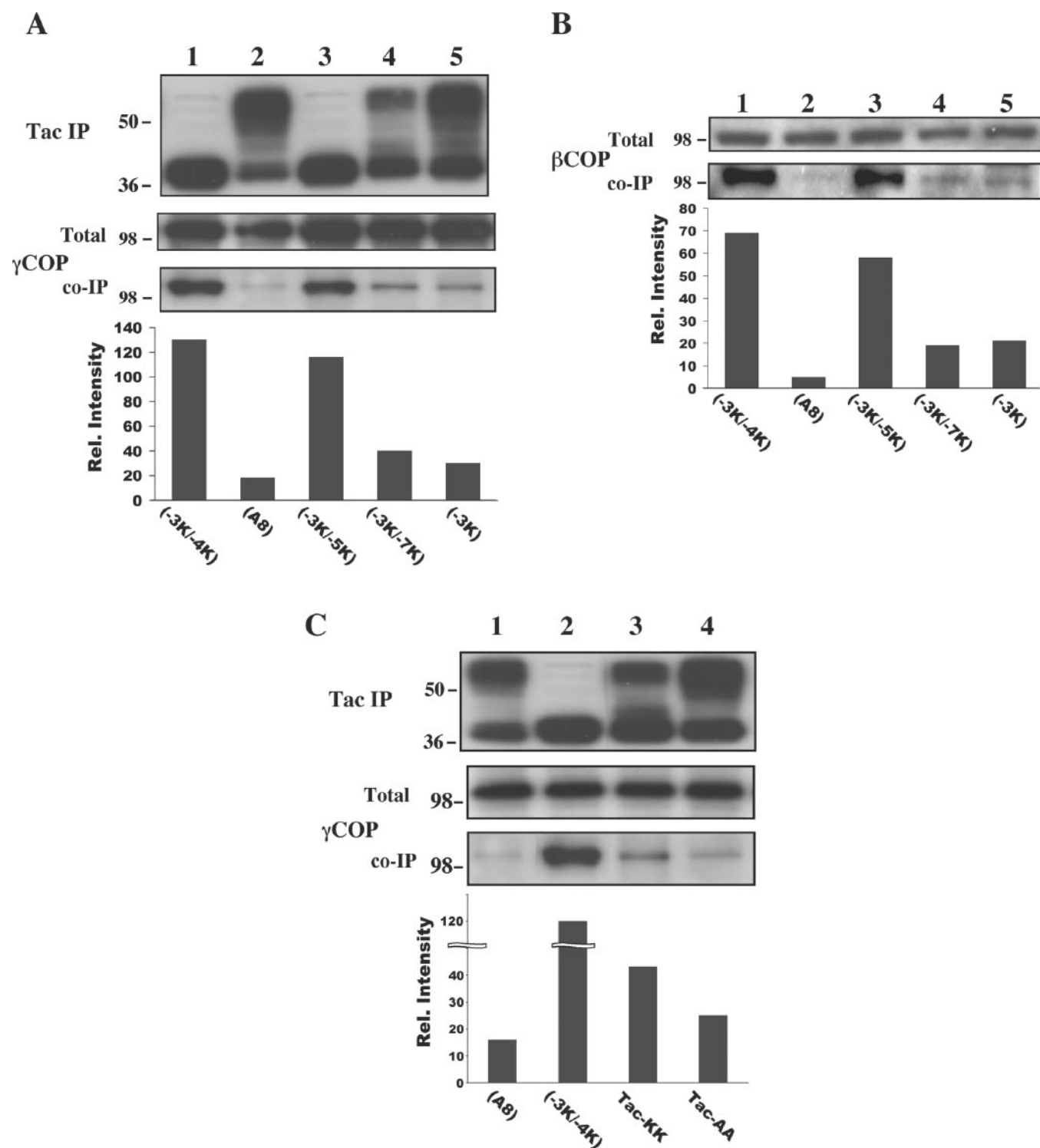


FIGURE 2. Determination of COPI association with Tac reporter molecules. A, HEK293 cells were transfected with either Tac-(−3K/−4K) (lane 1), Tac-A8 (lane 2), Tac-(−3K/−5K) (lane 3), Tac-(−3K/−7K) (lane 4), or Tac-(−3K) (lane 5), then immunoprecipitated with an anti-Tac Ab, and then immunoblotted using either an anti-Tac Ab (upper blot) or an anti- γ -COP Ab (bottom blot). Total γ -COP from each lysate was determined by immunoblot analysis of whole cell lysates (bottom two blots). The molecular mass markers are shown on the left. The visualized γ -COP from the bottom blot was scanned and analyzed using NIH Image version 1.63 as summarized in the accompanying bar graph. B, parallel immunoblots to those described in panel A (middle and bottom panels) were probed with an anti- β -COP Ab to reveal both total β -COP (middle panel) and co-immunoprecipitated β -COP (lower panel) for each transfectant followed by band analysis as described in panel A. C, HEK293 cells were transfected with Tac-A8 (lane 1), Tac-(−3K/−4K) (lane 2), Tac-PKPNPKNN (lane 3), and Tac-PAPNPANN (lane 4) and then analyzed as described in panel A. The data shown are representative of three different experiments.

Tac-AAAKKKAA, designated Tac-(−3K/−4K/−5K) or optimal surface expression (Tac-A8). As summarized for the analysis of quadruplicate transfections (Fig. 1B), Tac-KK showed an intermediate level of surface expression compared with Tac-(−3K/−4K) and Tac-(−3K/

−4K/−5K) and the Tac-A8 positive control. As before, differences in surface expression were not due to differences in protein expression as revealed in the Fig. 1B immunoblot. Taken together these data suggest that the −3/−7 dilysine residues are intrinsically less functional in con-

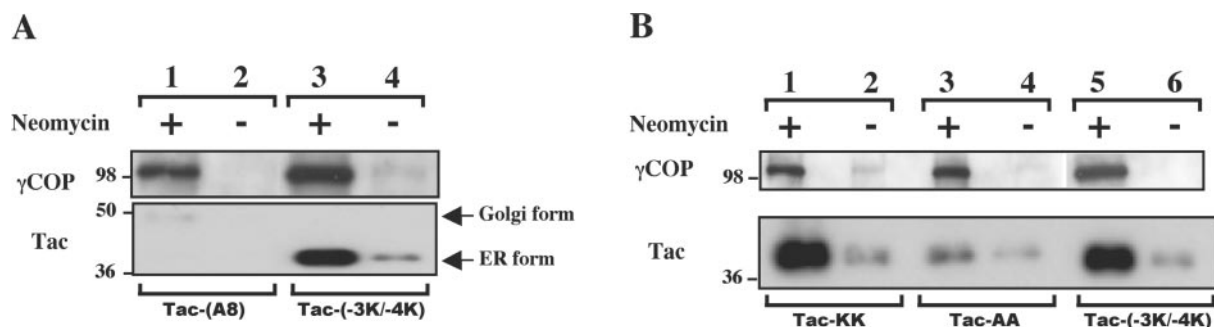


FIGURE 3. Neomycin co-precipitation of COPI and associated Tac reporter molecules. A, HEK293 cells were transfected with either Tac-A8 (lanes 1 and 2) or Tac(-3K/-4K) (lanes 3 and 4) and cell lysates then treated with or without neomycin to a final concentration of 1 mM to precipitate COPI complexes. Precipitated protein was fractionated by SDS-PAGE and immunoblotted using either γ -COP (upper blot) or Tac (lower blot) Abs. The position of the Tac reporter Golgi and ER forms and the molecular mass markers are indicated. B, Tac-PKPNPKNN (lanes 1 and 2), Tac-PAPNPANN (lanes 3 and 4) and Tac(-3K/-4K) (lanes 5 and 6) transfectant lysates were treated with (+) or without (-) neomycin and the resulting precipitates analyzed by immunoblotting as described in panel A.

trolling steady-state transport than the canonical KKXX- or KXXXX-COOH motifs.

To further evaluate the transport characteristics of the Tac-KK, Tac-AA, and Tac(-3K/-4K) reporter molecules, we compared the ER and Golgi localization of each transiently expressed molecule by confocal microscopy. Using calnexin as ER localization marker and giantin as a Golgi marker we observed that Tac(-3K/-4K) (Fig. 1C, middle row) was strongly localized to the ER as shown in the merged images of FITC-labeled anti-Tac Ab (first column) and Texas Red-labeled anti-calnexin Ab (second column, ER-merge). The Tac-KK reporter (top row) clearly showed a higher degree of ER localization than the Tac-AA reporter (bottom row). At the same time both reporters showed distinct localization to the Golgi compartment, as summarized in the merged image (Golgi-merge) of FITC-labeled anti-Tac and Texas Red-labeled anti-giantin Abs, as well as prominent cell-surface expression using FITC-labeled anti-Tac Ab.

Role of Fc ϵ RI α -Chain -3/-7 Lysine Residues in COPI Association—To determine the potential role of the Fc ϵ RI α -chain -3/-7 lysine residues in retrograde transport we first tested the capacity of various Tac reporter molecules (Tac(-3K/-4K), Tac-AAAKAKAA, designated Tac(-3K/-5K) and Tac-AKAAAKAA, designated Tac(-3K/-7K)) and Tac-AKAAAKAA (designated Tac(-3K)) to associate with COPI compared with the negative control reporter Tac-A8. We measured the relative association of the COPI γ - and β -subunits with each reporter molecule from transfected HEK293 cells by immunoprecipitation of detergent-solubilized protein with an anti-Tac Ab (Fig. 2A, top panel) followed by analysis of COPI subunit co-precipitation by SDS-PAGE and Western blotting using either an anti- γ -COP Ab (Fig. 2A, middle and bottom immunoblots) or anti- β -COP Ab (Fig. 2B). A similar amount of Tac-related protein and γ -COP protein were immunoprecipitated from each of the five transfections as shown in the upper and middle panels of Fig. 2A, respectively, whereas a distinctly greater amount of γ -COP co-precipitated with the Tac(-3K/-4K) and Tac(-3K/-5K) reporters (lanes 1 and 3, respectively). At the same time a low but discernable amount of γ -COP co-precipitated with either Tac(-3K/-7K) or Tac(-3K) compared with Tac-A8 as quantitated by densitometric analysis (Fig. 2A, bottom panel). A generally similar co-precipitation pattern was found for β -COP as shown in the anti- β -COP immunoblot (Fig. 2B) and as summarized in the accompanying densitometric analysis (Fig. 2B, bottom).

We then assessed the capacity of the Fc ϵ RI α -chain C-terminal sequence, expressed as Tac- α chimera, to promote association with COPI using the same immunoprecipitation strategy. Precipitation of Tac-A8, Tac(-3K/-4K), Tac-KK, and Tac-AA was followed by anti- γ -COP immunoblot analysis of gel-fractionated samples. As before sim-

ilar amounts of Tac-related protein and γ -COP were immunoprecipitated from each transfectant lysate as shown in the top and middle immunoblots of Fig. 2C, whereas the Tac(-3K/-4K) reporter showed the largest amount of γ -COP co-precipitation (bottom immunoblot, lane 2). Densitometric analysis showed that more γ -COP co-precipitated with Tac-KK than either Tac-AA or the Tac-A8 negative control but considerably less than co-precipitated with the canonical Tac(-3K/-4K)-positive control reporter (Fig. 2C, bottom panel), in general agreement with the data in Fig. 2A.

Aminoglycoside antibiotics such as neomycin have been previously shown to effectively precipitate COPI complexes (42, 43). As a potential way to corroborate our results showing α -chain sequence-dependent COPI association, we tested the capacity of neomycin to co-precipitate various Tac reporter molecules expected to show varying degrees of COPI association. Initial experiments tested whether neomycin could co-precipitate molecules known to have either strong (Tac(-3K/-4K)) or negligible (Tac-A8) COPI-binding activity. As shown in Fig. 3A, transfectant lysates treated with neomycin, but not without, readily precipitated γ -COP from both Tac-A8- and Tac(-3K/-4K)-transfectant lysates (top panel, lanes 1 and 3, respectively). However, only the Tac(-3K/-4K) reporter was co-precipitated (Fig. 3A, bottom panel, lane 3). We then tested the relative capacity of neomycin to co-precipitate Tac-KK compared with Tac-AA and observed a high level of both Tac-KK and Tac(-3K/-4K) precipitation in the same experiment (Fig. 3B, bottom panel, lanes 1 and 5, respectively) that was strikingly greater than the amount of co-precipitated Tac-AA (Fig. 3B, lane 3). In separate experiments we also compared the neomycin precipitation profile of the two mono-lysine Tac reporters (-3K and -7K) and found that the -3K reporter showed consistently more co-precipitation than the -7K Tac molecule that was at the same time significantly greater than negative control (data not shown). Taken together the neomycin co-precipitation results agree very well with our co-immunoprecipitation data (Fig. 2) and also corroborates that the -3 and -7 lysine residues contribute unequally in COPI association.

To more directly assess the role of the α -chain -3/-7 dilysine signal in controlling α -chain transport we examined the sub-cellular localization of wt α -chain compared with the -3/-7 dialanine α -chain mutant (K226A/K230A) in transfected cells. As shown in Fig. 4A, intracellular α -chain expression could be readily detected in permeabilized cells transfected with either wt α -chain or K226A/K230A. Further analysis revealed that the dialanine mutant showed extensive co-localization (merged image) with giantin, a marker for the cis/medial Golgi compartment (44), whereas the wt α -chain showed essentially no co-localization. The lack of Golgi localization of wt α -chain was consistent with our previous results showing

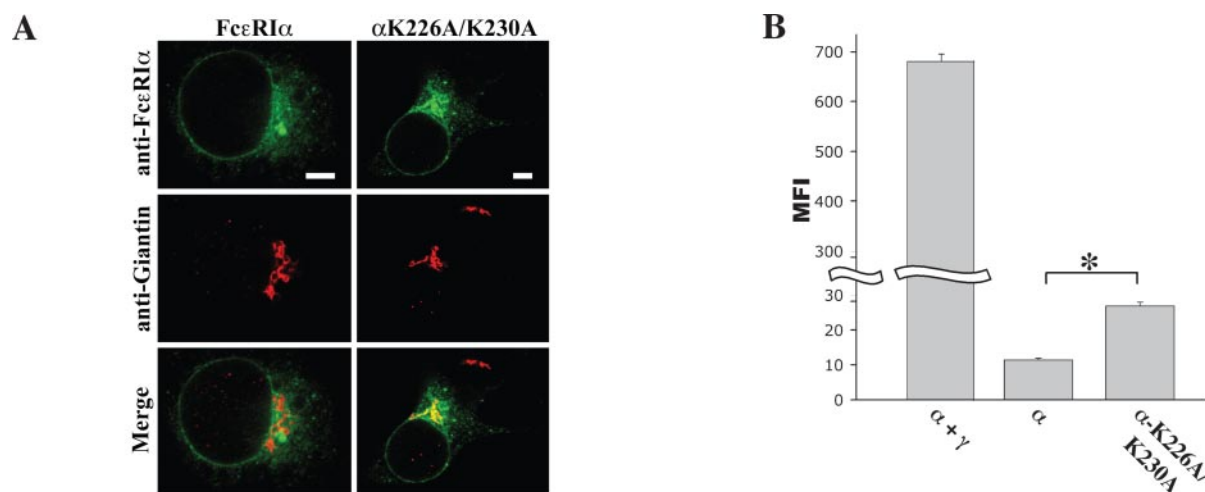


FIGURE 4. Substitution of FcεRI α-chain -3/-7 dilysine residues with alanine results in ER to Golgi transport but little surface expression. A, intracellular expression of human wt FcεRI α-chain and the -3/-7 dialanine mutant K226A/K230A in transfected COS-7 cells was visualized with an anti-FcεRIα mAb 15-1 and anti-giantin Ab with co-localization shown in yellow in the merged images. White scale bars represent 5 μm. B, FACS analysis from quadruplicate transfections (average MFI ± S.E.) of both wt α-chain and α-K226A/K230A. For comparison, the optimal expression of wt α-chain surface expression in cells co-transfected with human FcεRI γ-chain is shown. Statistical significance (*) was assigned based on a Student's *t* test *p* value of ≤0.05.

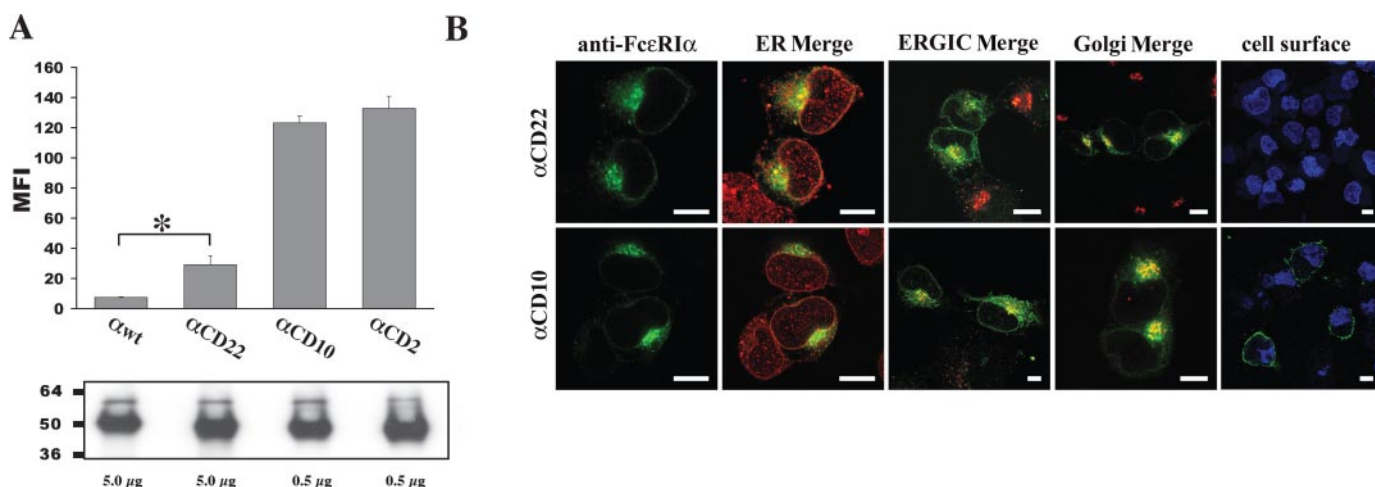


FIGURE 5. FcεRIα ER retention involves additional CD sequence. A, summary of the average MFI ± S.E. of surface expression for quadruplicate transfections of αCD22, αCD10, αCD2, and wt α-chain. B, analysis of αCD22 and αCD10 intracellular localization by confocal microscopy in transfected COS-7 cells. The first four columns show intracellular staining of the α-chain truncations (in green) and the middle three columns show the merged image (in yellow) from co-staining with either the ER marker calnexin, the Golgi marker giantin, or the anti-ER-Golgi Intermediate Compartment (ERGIC) marker ERGIC-53 (the latter three in red). The last column shows surface staining. Scale bars represent 10 μm.

that wt α-chain localizes exclusively to the ER in the absence of the FcεRI γ-chain (16). Analysis of quadruplicate K226A/K230A transfectants (Fig. 4B) revealed very low surface expression that was still significantly higher than wt α-chain ($p < 0.05$), although <5% of the optimal α-chain expression that occurs upon co-transfection of the FcεRI γ-chain (MFI = 600–700). Thus mutation of the -3/-7 dilysine signal clearly leads to enhanced α-chain transport characteristics but with little concomitant steady-state cell-surface accumulation.

Role of Additional FcεRI α-Chain CD Sequence in Controlling Steady-state Surface Expression—The cumulative evidence in this study suggests that the FcεRIα -3/-7 dilysine residues have relatively low function in controlling intracellular retention. Because the FcεRI α-chain is rigorously retained in the ER in the absence of assembly with the γ-chain, we hypothesized that additional α-chain sequence beyond the -3/-7 lysine residues might be involved in controlling α-chain trafficking. We first considered the possibility that additional sequence within the 33-residue CD might regulate α-chain cellular transport and assessed this possibility using different α-chain truncations. C-terminal

truncations containing either 2 (αCD2), 10 (αCD10), or 22 (αCD22) residues were transiently transfected in HEK293 cells followed by analysis of surface expression by flow cytometry and determination of sub-cellular localization characteristics by confocal microscopy. By FACS analysis both the αCD2 and αCD10 molecules were found to exhibit a pronounced level of surface expression compared with αCD22 as summarized in Fig. 5A. The low level surface expression of αCD22 was still significantly higher than wt α-chain ($p < 0.05$) and was generally comparable to K226A/K230A expression (see Fig. 4B). Consistent with the foregoing results, confocal microscopy analysis revealed that the αCD22 truncation mutant showed a much higher level of co-localization with the ER resident marker calnexin than αCD10 (ER Merge, Fig. 5B) and αCD2 (not shown). Further analysis showed that αCD22 and αCD10 co-localized to both the ERGIC and Golgi apparatus as shown in the merged images using antibodies to ERGIC-53 and giantin, respectively, with αCD10 showing a more prominent level of Golgi localization. Consistent with our FACS data αCD10, but not αCD22, showed a clear pattern of cell-surface expression. Taken together the pronounced differences in steady-state surface and intracellular expression between

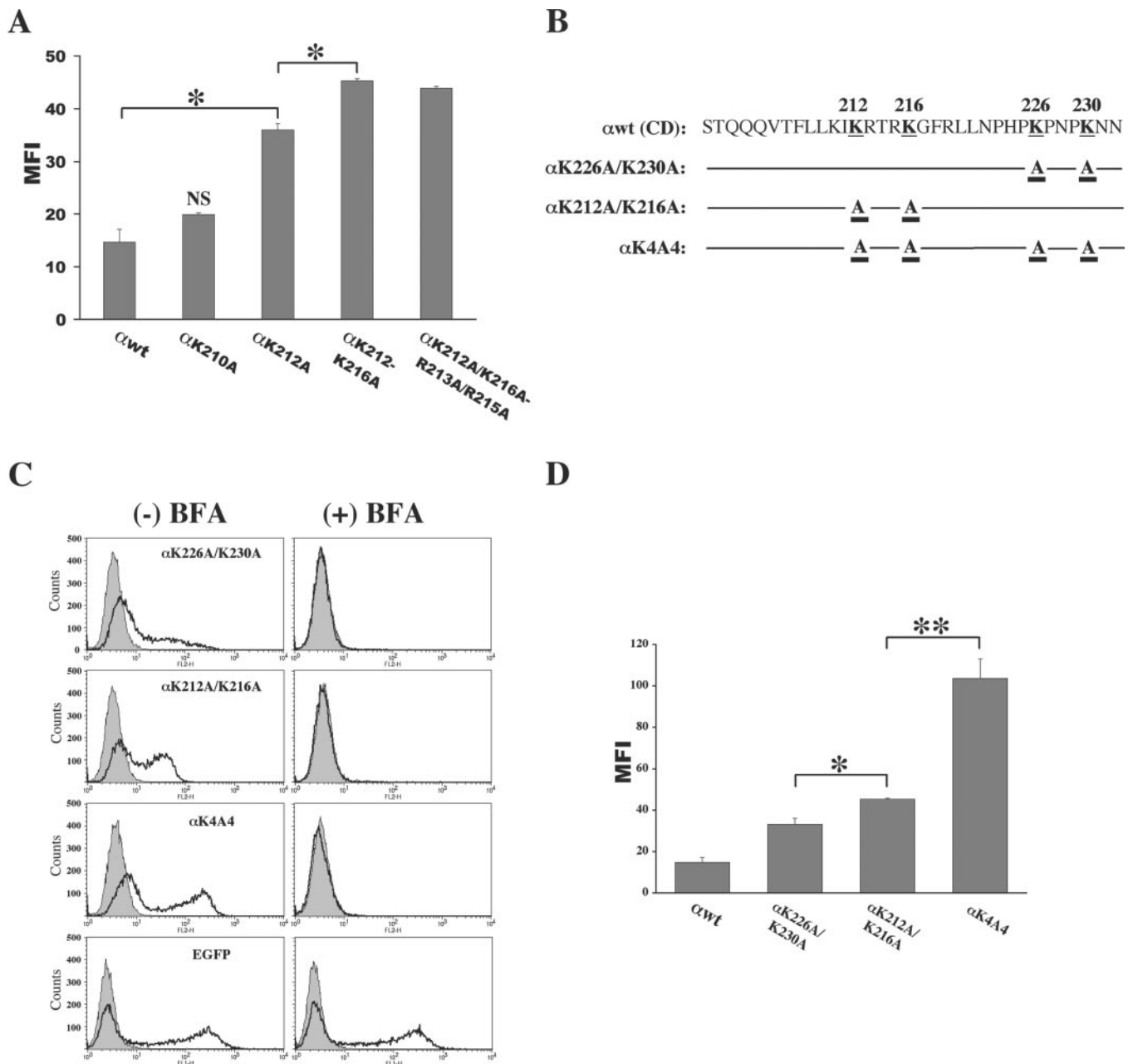


FIGURE 6. Alanine-scanning mutagenesis of FcεRI αCD residues. *A*, HEK293 cells were transiently transfected with either αwt, αK210A, αK212A, αK212A/K216A, or αK212A/K216A/R213A/R215A and analyzed for cell-surface expression by flow cytometry as described under "Materials and Methods." The average MFI ± S.E. from quadruplicate transfections is summarized. *B*, sequence comparison of the two dilysine to dialanine mutants (αK226A/K230A and αK212A/K216A) and the tetraalanine mutant designated αK4A4 (αK226A/K230A-K212A/K216A). *C*, cell-surface expression profile of mutant α-chain cells described in panel *B* (bold histograms) with or without BFA treatment prior to FACS analysis. For comparison surface expression of αwt (shaded histograms) and intracellular expression of a transfected enhanced green fluorescent protein reporter are shown. *D*, summary of average of MFI values ± S.E. for quadruplicate transfections from panel *C*.

αCD10 and αCD22 implicate an important role for the 12-residue sequence difference (KIKRTRKGFRL) between the two truncation mutants in controlling steady-state α-chain transport.

To further refine the sequence between αCD10 and αCD22 involved in α-chain retention, we compared the surface expression characteristics of additional α-chain truncations between αCD22 and αCD10 and observed that truncations made between αCD11 and αCD17 produced the greatest increase in cell-surface expression (data not shown), suggesting that the polybasic sequence Ile²¹¹-Lys-Arg-Thr-Arg-Lys²¹⁶ contains critical residues that function in controlling intracellular α-chain retention. To probe this region further we performed alanine-scanning mutagenesis targeting single or multiple residues within this region and

then compared the steady-state surface expression profile of each (Fig. 6A). From this analysis we determined that the two lysines at positions 212 and 216 contributed the greatest effect in regulating α-chain surface expression. We also assessed whether the internal diarginine sequence (²¹³RTR²¹⁵) might contribute to α-chain retention by comparing the surface expression of the αK212A/K216A mutant and the tetraalanine mutant αK212A/K216A/R213A/R215A and as summarized in Fig. 6A, no discernable difference in surface expression could be detected between the two mutant α-chains suggesting little if any role for the diarginine sequence in directing intracellular α-chain retention. Taken together our results reveal a novel dilysine sequence (Lys²¹²-Lys²¹⁶) that appears to function in regulating α-chain transport to the plasma membrane.

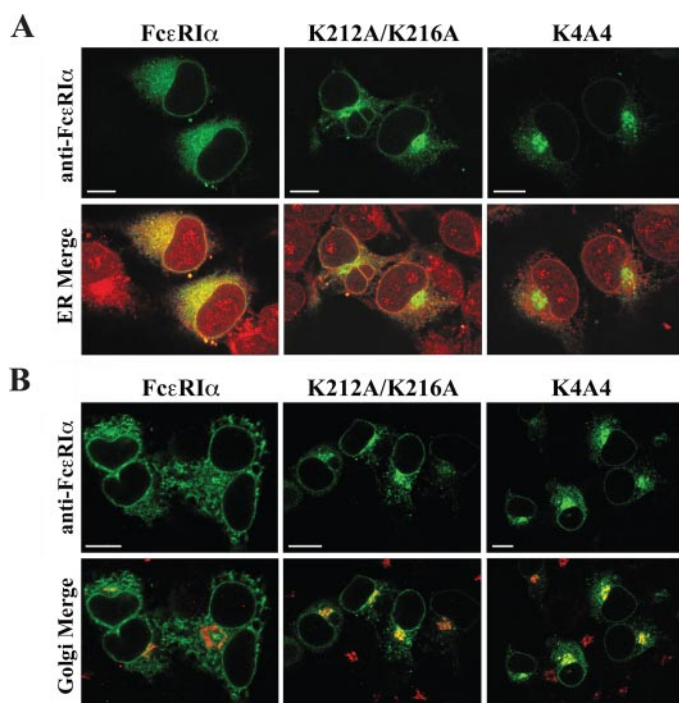


FIGURE 7. Analysis of the intracellular localization of FcεRI α-chain mutants αK212A/K216A and αK4A4. A, COS-7 cells were transfected with wt α-chain, αK212A/K216A, or αK4A4, and the permeabilized transfectants were stained with mAb 15-1 (green) and an anti-calnexin Ab (red). The two images were then merged to detect Ab co-localization (yellow) in the ER. B, the same transfectants were co-stained with 15-1 and anti-giantin Ab (red), and the two images were merged to visualize Golgi co-localization (also in yellow). Scale bars represent 5 μm.

Comparison of the Two FcεRIα Dilycine Signals in Regulating α-Chain Surface Expression—The relative capacity of the FcεRIα Lys²¹²–Lys²¹⁶ and Lys²²⁶–Lys²³⁰ dilycine residues to function in intracellular retention was then appraised in a side-by-side comparison of the surface expression efficacy of the corresponding dialanine α-chain mutants αK226A/K230A and αK212A/K216A (Fig. 6B). As shown in the representative FACS data summarized in Fig. 6C (left column, brefeldin A (–) BFA), each of the two double alanine mutants (*bold histograms*) showed higher surface expression than wt α-chain (*shaded histogram*) with the αK212A/K216A mutant showing significantly higher surface expression than αK226A/K230A (Fig. 6D, $p < 0.05$, $n = 4$) under conditions of comparable total protein expression (not shown). By comparison the corresponding tetraalanine mutant αK226A/K230A/K212A/K216A (designated αK4A4) showed the highest cell-surface expression that was also noted to be significantly greater than the sum of the MFI values for each double alanine mutant (Fig. 6D). Additionally we were able to conclude that the different mutants reached the cell surface via transit through the conventional early secretory pathway as judged by the finding that BFA treatment of transfected cells, a reagent that inhibits intracellular protein transport and receptor-surface expression (45–47), completely blocked surface expression of all α-chain molecules but without affecting cytosolic enhanced green fluorescent protein expression.

Intracellular Localization of αK212A/K216A, αK226A/K230A, and αK4A4 Mutants—To further investigate the role of the four lysine residues in intracellular α-chain retention, we compared the subcellular localization of αK212A/K216A and αK4A4 in permeabilized COS-7 cells by confocal microscopy. As before co-localization experiments were carried out using anti-calnexin and anti-giantin as ER and Golgi markers. The wt α-chain clearly exhibited a strong ER staining pattern as shown in the merged image of anti-FcεRIα mAb 15/1 and anti-

calnexin Ab staining (Fig. 7A), whereas little concomitant Golgi staining could be detected (Fig. 7B) indicating strict ER localization, as we have previously observed (16). By comparison, αK212A/K216A showed relatively little ER localization (Fig. 7A) but a significant degree of expression in the Golgi (Fig. 7B) compartment, as shown in the *ER Merge* and *Golgi Merge* images, respectively. In addition the αK212A/K216A mutant showed more prominent Golgi localization in a side-by-side comparison with the wt α-chain and the αK226A/K230A mutant (similar to Fig. 4A). Consistent with our other data, the αK4A4 mutant showed little detectable ER localization but prominent co-localization with the Golgi marker indicating facile ER to Golgi transport of the tetraalanine α-chain mutant.

Further Analysis of Lys²¹²–Lys²¹⁶-dependent Function—To further corroborate the functional capacity of the newly identified Lys²¹²–Lys²¹⁶ intracellular retention signal, we analyzed the expression characteristics of several chimeric proteins comprised of the extracellular segment and TMD of the Tac antigen combined with either the wt α-chain CD sequence (Tac-αCDwt) or mutant CD sequences (Tac-αCDK212A/K216A, Tac-αCDK226A/K230A, and Tac-αCDK4A4). We first analyzed surface expression characteristics by flow cytometry and found that the Tac-αCDwt reporter showed almost no cell-surface expression (Fig. 8A, *shaded histogram* in each panel) indicating that the α-chain CD is sufficient for intracellular retention. By contrast both the Tac-αCDK212A/K216A and Tac-αCD-K226A/K230A molecules showed significant surface expression with Tac-αCDK226A/K230A showing greater than 2-fold higher expression than Tac-αCDK212A/K226A (MFI = 120 and MFI = 50, respectively, in Fig. 8A). The most striking result was that the tetraalanine Tac reporter (Tac-αCDK4A4) showed the highest cell-surface expression (MFI > 400) further suggestive of a cooperative relationship between the two dilycine signals in regulating α-chain retention.

Because the Tac antigen contains two *N*-linked glycans (48), it is possible to readily distinguish between the ER and Golgi forms by gel migration differences and by endoglycosidase H (endo H) sensitivity (38). We analyzed the relative abundance of Golgi and ER forms of each transfected Tac reporter from Fig. 8A by treatment of each immunoprecipitated Tac-αCD molecule with endo H followed by SDS-PAGE fractionation and anti-Tac immunoblotting. For Tac-αCDwt, a band of ~44 kDa was detected prior to endo H treatment that was quantitatively converted to a 40-kDa band by endo H (Fig. 8B, *upper panel*) defining the 44-kDa species as the ER form of Tac-αwt and indicating that this was the only glycoform detectable for this reporter. For the other three Tac-αCD molecules, endo H-resistant glycoforms characteristic of Golgi-derived processing were detected and included a 54- to 60-kDa pattern present in all three Tac-α reporters (*upper arrow* in Fig. 8B) as well as a 44- to 54-kDa glycoform pattern (*lower arrow* in Fig. 8B) that was far more evident in the K212A/K216A and K4A4 mutants, apparently reflecting more heterogeneous and relatively less *N*-glycosylation of these mutants. After densitometric scanning, we quantitated the ratio of the cumulative Golgi glycoforms to the ER form for each reporter (Fig. 8B, *bar graph*) and, consistent with the FACS data in panel A, found that the Tac-αCDK4A4 molecule showed the highest Golgi to ER ratio. At the same time a higher level of Golgi processing was observed for the Tac-αCD-K226A/K230A compared with Tac-αCD-K212A/K226A that also paralleled the surface expression results (Fig. 8A).

To extend the foregoing analysis we explored early biosynthesis kinetics of the same reporters by pulse-chase and SDS-PAGE band analysis. Prior to chase all ³⁵S-labeled Tac-αCD proteins migrated as the characteristic ER form (44 kDa) and were expressed at comparable levels (Fig. 8C). The Golgi-processed forms of the Tac-αCDK226A/K230A and Tac-αCDK4A4 were clearly evident after 1 h of chase, whereas

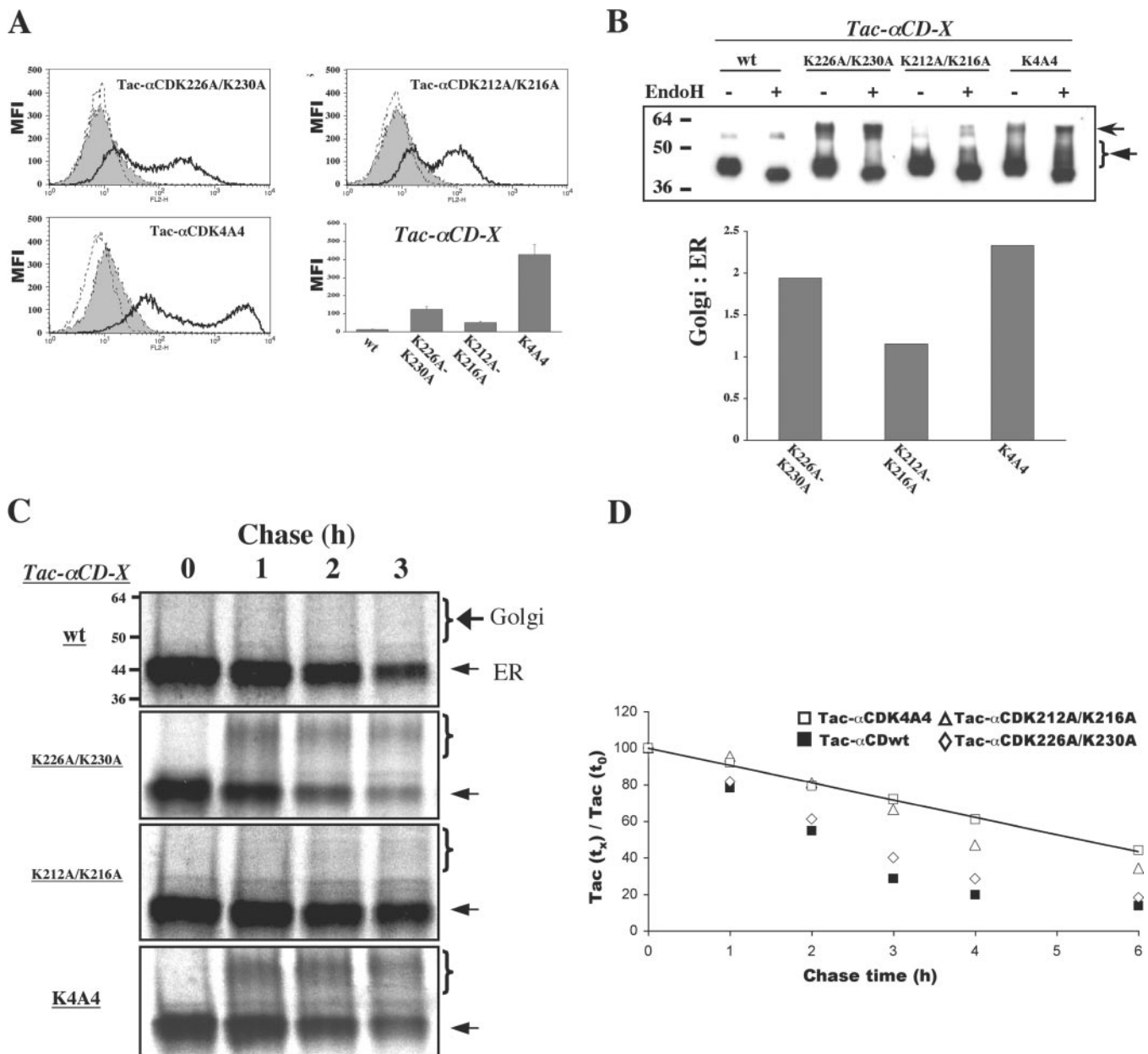


FIGURE 8. Transport properties and biochemical analysis of chimeric Tac- α reporter molecules. A, representative FACS profiles are shown for cell-surface expression of Tac- α CDK226A/K230A, Tac- α CDK212A/K216A, and Tac- α CDK4A4 constructs (**boldface histograms**) compared with both Tac- α CD(wt) (**shaded histograms**) and isotype control staining (**dashed histograms**). MFI values $\pm$ S.E. from quadruplicate transfections are summarized in the **bar graph**. B, HEK293 cells were transfected with the Tac constructs described in **panel A** followed by anti-Tac immunoprecipitation, endoglycosidase H treatment or not, and immunoblotting. Two endo H-resistant Golgi forms were detected, a 54- to 60-kDa pattern (**upper arrow**) and a 44- to 54-kDa pattern. The ratio of the cumulative Golgi forms to ER forms was calculated and normalized to the ratio of Golgi to ER forms of endo H-treated wt α -chain (set to 1.0). C, COS-7 cells were transfected with the Tac- α reporters described in **panel A**, pulsed with [^{35} S]Met, chased for the indicated times, immunoprecipitated, and analyzed by SDS-PAGE and autoradiography. The ER and Golgi forms of the Tac reporters are indicated. D, graphical summary of the amount of immunoprecipitated Tac- α chimeric protein (ER plus Golgi forms: Tac(t_x)) at each chase time (t_x) and expressed as the percentage of the initial amount of each prior to chase initiation (Tac(t_0)). Tac- α CDwt (**closed box**), Tac- α CDK226A/K230A (**diamond**), Tac- α CDK212A/K216A (**triangle**), and Tac- α CDK4A4 (**open box**). The data are representative of results from three replicate experiments.

glycosylated Tac- α CDK212A/K216A appeared to accumulate more slowly and to a lesser extent, although still greater than Tac- α CDwt. We then compared the total protein expression (sum of the ER plus Golgi-processed forms) for each ^{35} S-labeled chimeric Tac- α CD molecule remaining over a 6-h chase period. When expressed as the percentage of initial Tac- α CD protein prior to chase initiation (Fig. 8D), it was apparent that greater amounts of Tac- α CDK4A4 (**open box**) and Tac- α CDK212A/K216A (**triangle**) were produced compared with either Tac- α CDwt (**closed box**) or Tac- α CDK226A/K230A (**diamond**) suggesting the possibility of enhanced protein stability of the former two molecules. Interestingly, the most highly expressed reporter, Tac- α CDK4A4,

also appeared to degrade in a linear fashion over time ($R^2 = 0.997$). Total Tac- α CDK212A/K216A expression was clearly higher than Tac- α CDwt suggesting that one or both residues of the Lys²¹²–Lys²¹⁶ sequence are linked to enhanced α -chain protein degradation. On the other hand the total Tac- α CDK226A/K230A expression was comparable to Tac- α CDwt suggesting that the Lys²²⁶–Lys²³⁰ residues play a lesser role in α -chain degradation.

The Single, Charged Residue in the α -Chain TMD Contributes to α -Chain Cellular Transport—A role of the Fc ϵ RI α TMD in α -chain surface expression has been previously suggested (49) and we hypothesized that this region might function cooperatively with one or more of

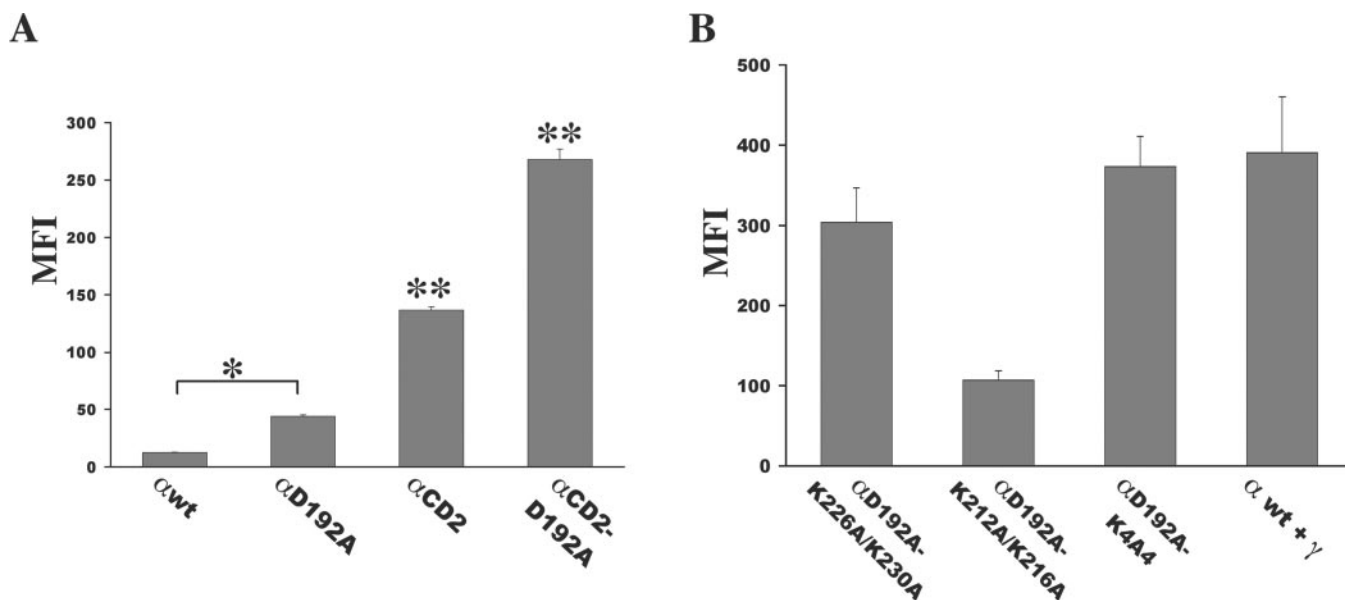


FIGURE 9. Contribution of the TMD-charged residue (Asp¹⁹²) in α -chain cellular transport. A, HEK293 cells were transfected with α wt, α D192A, α CD2, and α CD2-D192A and analyzed for cell-surface expression by flow cytometry. The average MFI $\pm$ S.E. for quadruplicate transfections is summarized. Statistical significance was assigned as follows: *, p values ≤ 0.05 ; **, p values ≤ 0.001 . B, FACS analysis of α D192A-K226A/K230A, α D192A-K212A/K216A, or α D192A-K4A4 transfectants compared with the co-transfection of α wt plus Fc ϵ R1 γ analyzed as described in panel A.

the CD dilysine retention signals. We first tested the effect of mutating the lone charged residue within the TMD (D192) on surface expression compared with wt α -chain and the α -chain truncation mutant lacking all but 2 CD residues (α CD2). As summarized in Fig. 9A, the α D192A mutant showed significantly higher surface expression than wt α -chain but still considerably less than α CD2 suggesting an unequal role of the α -chain CD and TMD (D192) in intracellular retention. In addition, the double mutation, α CD2/D192A, showed the highest surface expression suggesting a cooperative effect between the two determinants. We extended this analysis to α -chain mutants containing lysine to alanine mutations and as summarized in Fig. 9B both the α D192A/K226A/K230A and α D192A/K212A/K216A mutants showed higher surface expression than α D192A with the former mutant showing nearly 3-fold higher expression than the latter. Expression of α D192A-K4A4 revealed an essentially additive effect between the two dilysine sequences and the Asp¹⁹² residue. Furthermore the level of expression of α D192A-K4A4 was indistinguishable from cells co-transfected with wt α - and γ -chains suggesting that the mutagenized residues in the α D192A-K4A4 molecule comprise essentially the complete intracellular retention signal of the Fc ϵ R1 α -chain.

DISCUSSION

It has recently been shown that assembly of the multimeric Fc ϵ R1 $\alpha\beta\gamma$ complex is an important step in regulation of Fc ϵ R1 surface expression that in turn is closely linked to the magnitude of the allergic response (17). However, comparatively little is known about the molecular signals that regulate trafficking of the individual Fc ϵ R1 subunits or the 2 Fc ϵ R1 isoforms, $\alpha\gamma$ 2 and $\alpha\beta\gamma$ 2, through the early secretory system. We have previously shown (16) that *N*-linked core glycosylation of the nascent Fc ϵ R1 α -chain is an important checkpoint in ER quality control (50). However, the only Fc ϵ R1 sorting signal described thus far is a -3/-7 dilysine ER retention/retrieval signal situated near the C terminus of the α -subunit (18). In this work we re-evaluated the role of the -3/-7 dilysine motif in steady-state α -chain surface expression by first expressing variants of the C-terminal α -chain sequence PKPNPKNN fused to the C terminus of the Tac (CD25) membrane protein. We

found that both Tac-PKPNPKNN and the corresponding -3/-7 dialanine variant showed significant cell-surface expression (Fig. 1, A and B) with the latter also showing prominent Golgi localization (Fig. 1C). Because dilysine signals are known to direct ER localization by retrieval from the Golgi through interaction with the multimeric COPI complex (25–27, 51), we tested whether the Fc ϵ R1 α -chain C-terminal sequence might show COPI association characteristics in an immunoprecipitation assay. Comparison of Tac-PKPNPKNN to a Tac reporter (Tac(-3K/-4K)) bearing a canonical KKXX-COOH ER retention signal demonstrated that the α -chain -3/-7 dilysine signal binds a relatively low but discernable amount of COPI (Fig. 2), a finding further validated using a neomycin-dependent COPI precipitation assay (Fig. 3). The foregoing results prompted us to explore the extent of steady-state expression of a -3/-7 dialanine α -chain substitution mutant. Interestingly the K226A/K230A dialanine mutant was found to show a very clear pattern of Golgi localization (Fig. 4A) but with little concomitant surface expression (Fig. 4B). Taken together our results suggest that the C-terminal α -chain sequence PKPNPKNN has a relatively low propensity to direct intracellular retention and may function uniquely in delaying ER to Golgi transport in a manner that is manifested by reduced cell-surface expression characteristics. The existence of functional -3/-7 dilysine ER retention/retrieval signals in other membrane proteins appears to be rare. For example, although nearly 40% of all cataloged ER membrane proteins include at least two basic residues (including arginine or histidine) at positions -2, -3, -4, and/or -5 (30), a search of 324 ER proteins in the Hera data base revealed only nine with a lysine situated at position -3 but none with a -3/-7 dilysine sequence (30).

As noted earlier the Fc ϵ R1 α -chain is rigorously retained in the ER in the absence of assembly with the γ -chain (16), and this phenotype is most likely derived from the action of stringent ER retention/retrieval activity. Because our data suggest that the C-terminal α -chain sequence has only relatively low ER retention/retrieval activity, we explored the possibility that additional α -chain sequence might also function in this capacity. Using α -chain CD truncation mutants we determined that the region defined by the sequence difference between α CD10 and α CD22, KIKRTRKGFRLL, func-

tions to promote intracellular retention (Fig. 5). Analysis of additional α -chain truncation mutants allowed us to identify a role for the two lysine residues at positions 212 and 216 in regulating α -chain surface expression. Comparison of the surface expression efficacy of the double alanine mutants α K212A/K216A and α K226A/K230A showed that both mutants were significantly expressed on the cell surface (Fig. 6, C and D) and in a manner that was completely blocked in the presence of BFA indicating that transport of each mutant occurred through conventional ER to Golgi trafficking. In addition the α K212A/K216A mutant showed enhanced Golgi localization compared with either the wt α -chain (Fig. 7B) or α K226A/K230A (Fig. 4A). Moreover, co-mutation of the Lys²¹²/Lys²¹⁶ and the Lys²²⁶/Lys²³⁰ residues (α K4A4) afforded much higher cell-surface expression in transfected cells than either of the two dialanine mutants (Figs. 6 and 8) suggesting a synergistic effect of the four lysine residues in regulating α -chain transport.

How a protein is retained or retrieved in the ER has been the focus of many studies over the last two decades. Accumulation of type I membrane proteins containing C-terminal dilysine motifs within the ER is generally thought to be caused by continuous retrieval from the Golgi apparatus (25) supported by the COPI protein complex (26–28), although a role for direct retention has also been shown (52, 53). In addition to dilysine and diarginine motifs, several other cytosolic sequences enriched in arginine and/or lysine have been shown to be involved in intracellular retention and, like the RXR motif, these sequences are typically located in a membrane-proximal region. RXR-like motifs have been shown to participate in retention of individual subunits of multimeric receptors such as the GluR5 (54) and the KA2 subunits of kainate receptor (55). Recently, an arginine-lysine motif (RK) involved in ER retention has been described in the α -subunit of the nicotinic acetylcholine receptor and has been shown to interact with γ -COP (35), a component of the COPI complex. In addition, membrane-proximal polylysine signals have now been identified that appear to function in ER to Golgi trafficking. For example in yeast a $-4/-6$ dilysine moiety in the p24 protein, Erv25p (56), a $-8/-9$ dilysine sequence in Rer1p (57), and the membrane proximal polylysine (KQKK) sequence in the GDP-mannose transporter (58) have been shown to mediate association with COPI subunits.

In addition to cytoplasmic motifs, it is now well established that TM determinants can also contribute to both ER retention (38, 59) and subunit oligomerization of some multimeric membrane receptors such as the T cell receptor α -subunit (38, 60), the CD8 co-receptor (61) and the nicotinic acetylcholine receptor (39). In many cases TM determinants have been identified as charged amino acids, and their role in targeting proteins for intracellular retention has been described (62–64). In this study we demonstrate that the α -chain TMD aspartate residue exerts an important role in controlling α -chain surface expression in combination with the intracellular retention function of the dilysine signals Lys²¹²–Lys²¹⁶ and Lys²²⁶–Lys²³⁰ (Fig. 9). Many proteins contain multiple trafficking determinants that often include both forward transport and ER localization signals (65). In addition some membrane proteins contain multiple ER retention signals with one signal typically localized to the TMD and the other frequently situated near the C terminus. The existence of multiple retrograde trafficking signals in the cytosolic portion of proteins is relatively less common. A recently reported example is the GDP-mannose transporter that cycles between the Golgi and ER compartments and is retrieved to the ER by association with a COPI subunit (Ret2p) via a cooperative contribution from both a membrane-proximal polylysine (KQKK) sequence and a C-terminal dibasic (RK) sequence (58). Our data reveal a novel mechanism of ER retention for a type I membrane protein that involves two different

dilysine motifs in the CD together with a TMD signal that appear to function synergistically to promote intracellular retention.

The presence of multiple ER retention/retrieval signals in the Fc ϵ RI α -chain suggests that there may be either redundancy in function between the signals or that all of the signals are necessary for presentation of a complete intracellular retention phenotype. The former possibility does not seem likely, because each of the three retention signals appears to function relatively inefficiently in ER retention. Therefore a more likely possibility is that the multiple retention signals function together to exert an overall highly stringent ER retention/retrieval signal. At present it is not known if assembly of the trimeric $\alpha\gamma 2$ complex fully masks the complete ER retention/retrieval signal of the α -chain or whether the $\alpha\gamma 2$ complex still expresses a partly functional ER retention signal. In this hypothetical situation multiple retention signals in the α -chain might facilitate not only the stepwise assembly of the $\alpha\gamma 2$ receptor but also the Fc ϵ RI $\alpha\beta\gamma 2$ complex in a manner somewhat analogous to the TCR-CD3 receptor complex that has at least one ER retention signal in each of its six subunits and only achieves optimal surface expression upon assembly of the complete complex (31). One prediction from this hypothesis is that the $\alpha\gamma 2$ complex might exhibit a measurable accumulation in the ER. In fact several studies have now shown that the ER form of the α -chain is produced at significantly higher levels in cells transfected with both α - and γ -chains compared with α -/ γ -/ β -transfectants (4, 66). The β -chain is well known to function in the amplification of Fc ϵ RI surface expression, and it has been suggested that it may act as a chaperone for Fc ϵ RI transport primarily because of its capacity to enhance post-translational Golgi processing of the α -chain (66). The molecular basis of this effect has not been determined, but the possibility remains that assembly with the β -chain in the ER results in reduced ER retention activity of the $\alpha\beta\gamma 2$ complex in direct analogy to the steric masking mechanism invoked to explain the transport competency of the Fc ϵ RI $\alpha\gamma 2$ complex (18). Such a role for the β -chain infers the likelihood of a direct α - β interaction, and previous studies (4, 17) using heterologous expression systems have now demonstrated the existence of α - β complexes. Of particular interest was the observation that α - β complexes could undergo a significant level of Golgi processing as shown by the formation of endo H-resistant α -chains (17), a finding indicative of escape of α - β heterodimers from the ER that in turn implicates a role for the β -chain in at least partial masking of the α -chain ER retention signal. Additional experiments are now in progress to test differential transport properties of trimeric *versus* tetrameric Fc ϵ RI complexes.

In conclusion this study demonstrates that α -chain intracellular retention is controlled by a multicomponent signal involving a charged residue in the TMD and two CD sequences. In addition all three components appear to function in a synergistic manner to stringently retain the Fc ϵ RI α -subunit in the ER prior to assembly with other Fc ϵ RI subunits. As described for many multimeric receptors, these retention signals become masked during assembly allowing only cell-surface expression of functional receptors, although it remains an open question whether the Fc ϵ RI $\alpha\gamma 2$ isoform shows intrinsically lower surface expression than the tetrameric $\alpha\beta\gamma 2$ isoform due to a hypothetical exposure of part of the overall α -chain retention signal in the $\alpha\gamma 2$ complex. The experiments described in this study were facilitated by the use of a high efficiency transfection system, and it remains to be determined if α -chain transport characteristics are similar in mast cells. To address this issue we are presently devising new studies using γ -chain-deficient mast cells expressing human Fc ϵ RI α -chain mutants to study γ -chain-independent transport properties of the human α -chain in the context of cells that normally express Fc ϵ RI.

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