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Clathrin-mediated Endocytosis of Mammalian Erythroid AE1 Anion Exchanger Facilitated by a YXXΦ or a Noncanonical YXXXΦ Motif in the N-Terminal Stretch

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ABSTRACT. To explore the roles of the conserved YXXΦ-type motif in the erythroid-specific N-terminal stretch of anion exchanger 1 (AE1), cell surface expression and internalization of various mutants derived from murine erythroid AE1 tagged with an N-terminal enhanced green fluorescent protein and an extracellular FLAG (EGFP-mAE1Flag) were explored in K562 and HEK293 cells. EGFP-mAE1Flag showed rapid internalization, in association with the internalizations of transferrin and the endogenous AE1 chaperone-like protein glycophorin A in K562 cells. Disruption of the conserved Y72VEL sequence markedly reduced the internalization and increased the relative abundance of cell-surface AE1, whereas substitution of the N-terminal region from bovine AE1 that lacks the relevant motif for the corresponding region had less of an effect on internalization. Deletion or substitution mutations of the Y7EDQL sequence in the bovine N-terminal stretch resulted in the decreased internalization of the AE1 proteins. Cell surface biotinylation and deglycosylation studies showed that approximately 30% of the cell-surface EGFP-mAE1Flag and several other mutants was sorted to the plasma membrane without *N*-glycan maturation in the Golgi apparatus. These findings indicate that the conserved YXXΦ sequence or a noncanonical YXXXΦ sequence in the N-terminal region facilitates the endocytic recycling of erythroid AE1 through a clathrin-mediated pathway.
KEY WORDS: anion exchanger 1 (AE1), endocytic recycling, endocytosis, erythroid, YXXΦ motif.

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Anion exchanger 1 (AE1, also called band 3 in red cells) is an integral membrane protein expressed in red cells and kidneys [4, 23]. The critical roles of AE1 in the maintenance of red cell shape and stability and in acid-base homeostasis are found in disease phenotypes due to various AE1 gene (*SLC4A1*) mutations [11, 14, 21]. Pathogeneses of several AE1 anomalies involve defects in the trafficking and polarized sorting of mutant polypeptides [4, 12, 28]. Moreover, the expression of AE1 in the plasma membrane plays a key role in the organization of the membrane skeleton during erythroid cell maturation, as well as that of some other proteins involved in linking the lipid bilayer to the membrane skeletal network [5, 15]. However, the regulatory mechanisms for AE1 trafficking remain unclear.

Sequence motifs located within the cytoplasmic domains participate in the plasma membrane expression, polarized sorting, endocytosis, and recycling of transmembrane proteins. One of the best characterized signals is the tyrosine-based YXXΦ-type sorting signal, where X is any amino acid residue and Φ is a bulky hydrophobic residue [26] (The single letter abbreviations for amino acid residues are used throughout the present study.). Previous studies have shown that the YXXΦ motif in the C-terminal tail (Y904DEV) is essential in the basolateral sorting of human kidney AE1. A deletion mutation of this region can cause distal renal tubular acidosis resulting from the mislocalization of kidney AE1 [7, 24]. However, the mutant AE1 is clearly able to reach the red cell surface of patients [25], which suggests that the renal and erythroid AE1 proteins utilize different signals in cell surface expression.

On the other hand, a kidney-specific variant of chicken AE1 (AE1-4) has been shown to use the YXXΦ motif in the N-terminal region (Y47VEL) for polarized sorting and Golgi recycling [2, 3]. It should be noted that several different variants of chicken erythroid AE1 have the same peptide segment containing the Y47VEL sequence [6] and exhibit internalization and recycling to the Golgi after initial sorting to the plasma membrane possibly through clathrin-mediated endocytosis [9]. The erythroid AE1 proteins in various mammals, including humans, possess the N-terminal stretch that comprises less than 85 amino acid residues and is absent in the kidney isoforms. Despite the variety of amino acid sequences, the YVEL sequence corresponding to Y47VEL in chicken AE1 is well conserved among various mammalian species (Fig. 1).

These findings suggest that the N-terminal YXXΦ-type sequence plays some role in the membrane trafficking and/or recycling of AE1 in mammalian erythroid cells as reported in chicken AE1 variants. In this context, it is interesting that bovine AE1 has the C68VQL sequence instead of the conserved YVEL sequence. Our previous study showed that bovine AE1 is stable in the plasma membrane when

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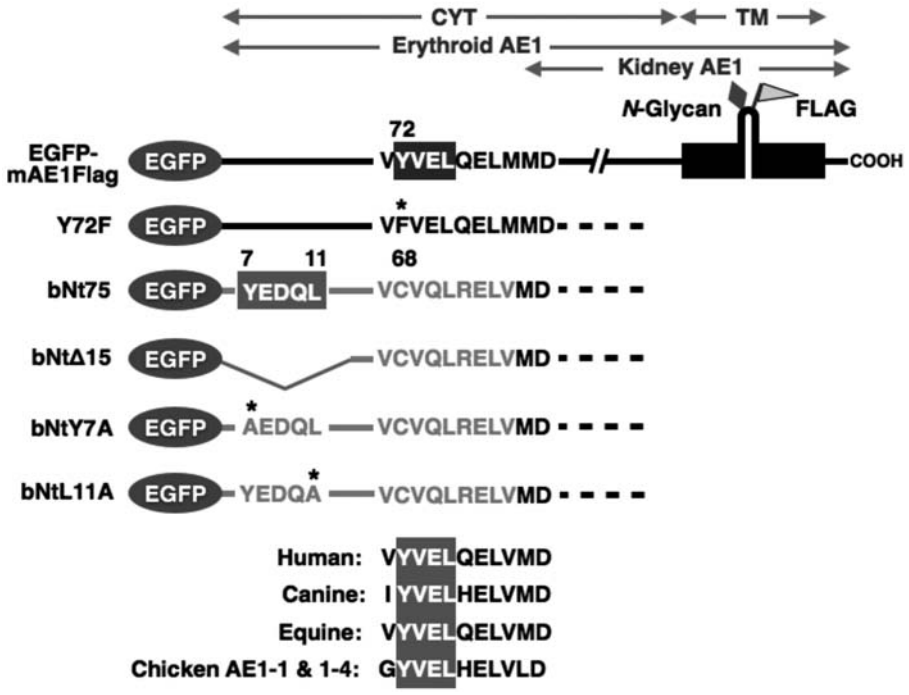

Fig. 1. Schematic illustration for EGFP- and FLAG-tagged murine AE1 mutants. Mammalian AE1 consists of the cytoplasmic domain (*CYT*), transmembrane domain (*TM*), and a short cytoplasmic C-terminal tail. Erythroid AE1 has the *N*-terminal stretch sequence that is absent in kidney AE1. The EGFP-mAE1Flag contains EGFP and FLAG tags that were added to the N-terminus and to the C-terminal side of the *N*-glycosylation site (N660) in the 4th extracellular loop of murine AE1 (GenBank ID: NM_011403), respectively. In the bNt75 mutant, the N-terminal region is replaced with that of bovine AE1 (GenBank ID: NM_101836), shown in gray letters. The deletion mutant bNtΔ15 lacks the N-terminal 15 residues, including the Y7EDQL sequence. Asterisks indicate substituted amino acid residues in Y72F, bNtY7A and bNtL11A mutants. For reference, the sequences around the conserved YXXΦ-type motif (YVEL) in the N-terminal stretches from human, canine, equine and chicken AE1 (GenBank IDs: NM_000342, AB242566, AB242565, and NM_205522, respectively) are shown at the bottom. The conserved YVEL sequence and the YEDQL sequence characteristic to bovine AE1 are highlighted.

expressed in K562 cells [12].

The purpose of the present study was to clarify whether the N-terminal YXXΦ motif or some alternative sequence has roles in the sorting of mammalian erythroid AE1. To address this question, we prepared several AE1 mutants that have the murine polypeptide backbone and cytoplasmic and extracellular tags (enhanced green fluorescent protein [EGFP] and FLAG, respectively) for detection. We examined the membrane expression and internalization of these mutants in K562 erythroleukemia cells because K562 cells constitute a suitable expression system for AE1 as reported previously [12]. We then analyzed the internalization of several mutants with further mutations in HEK293 cells to determine the sequence that plays a role in the internalization of bovine AE1.

MATERIALS AND METHODS

Antibodies: The antibodies used were Cy3-labeled anti-FLAG M2 and anti-β-actin (Sigma Chemical Co.), anti-GFP (MBL, Nagoya, Japan), anti-glycophorin A (GPA) (Biogenesis Ltd.), anti-GM130 (BD Biosciences), anti-Rab11 (Cell Signaling Technology), and anti-flotillin-1 (BD Transduction Laboratories). Secondary antibodies labeled with Alexa-Fluor-405 or -568 were from Molecular Probes.

Construction of cDNA clones: Murine AE1 cDNA (GenBank ID: NM_011403) was described previously [1]. The FLAG tag sequence (H_2N-DYKDDDDK-COOH) was introduced between the 664th arginine and 665th glycine in the 4th extracellular loop of murine AE1 (Fig. 1) by inserting the FLAG epitope-coding DNA fragment into the *Xma* I site of the cDNA. The fragment was prepared by annealing the oligonucleotides with the sequences of 5'CCGGATGGATTACAAGGATGACGACGATAAGGG-3' (sense) and 5'-CCGGCCCTTATCGTCGTCATCCTTGTAATCCAT-3' (antisense). The FLAG-tagged murine AE1 cDNA was subcloned into pEGFP-C1 or a retroviral vector pLEGFP-C1 (both from Clontech) for expression of murine AE1 with EGFP and FLAG tags (EGFP-mAE1Flag). A substitution mutant Y72F EGFP-mAE1Flag (Fig. 1) was generated

by site-directed mutagenesis as described previously [12] using the antisense primer 5'-CCACACCGCACCCAAGCTCCGGTCAGGTC-3'. The bNt75 EGFP-mAE1Flag possessing the N-terminal sequence of bovine AE1 (bNt75) was created by replacing the N-terminal 79 amino acid residues of murine AE1 with the N-terminal 75 residues of bovine AE1 (GenBank ID: NM_101836) [11, 12]. Deletion mutants bNtΔ50, bNtΔ29, and bNtΔ15 were prepared by PCR amplification using the sense primers 5'-ACAGACACAGACTACCACACCACATCGCAA-3' (nt 151→180 with respective to the A of the initiation codon) for bNtΔ50, 5'-ATGGAAGAGGCAGAAGGTGACACAATTCAG-3' (nt 88→117) for bNtΔ29, and 5'-GAGCAACCGGAATATGAAGACCATGATTCC-3' (nt 46 →75) for bNtΔ15, respectively, in combination with the antisense primer 5'-AAGAGGCTCCAGAAGTTGAGGTAAG-3' (nt 329←353). Substitution mutants bNtY7A and bNtL11A were prepared by PCR amplification using the primers 5'-GCTGAAGATCAACTGGAGGAGGACTCTAGA-3' (sense, nt 19→47) and 5'-CTCCTCCGGATCCCCCATGGCGGCTGTCCTG-3' (antisense, nt -11 ←19) and site directed mutagenesis using the mutagenic primer 5'-GCGGAGGAGACTCTAGAGCAAAAGGAATAT-3' (nt 31→60), respectively.

Cell culture and expression of proteins: K562 and HEK293 cell culture, the transfection of HEK293 cells with plasmid vectors, and the infection of K562 cells with retroviruses were carried out as previously described [12].

Analyses of proteins: Preparation of the cell lysate, SDS-PAGE, immunoblotting, cell surface biotinylation, deglycosylation and fluorescence microscopy were performed as described previously [1, 12]. Quantification of signal intensities in immunoblotting was carried out by densitometric scanning as described previously [12] and are expressed as the means ± S.D.

Analysis of internalization of cell-surface proteins: HEK293 cells grown on collagen-coated coverslips and K562 cells were washed with ice-cold phosphate-buffered saline (PBS). Cells were stained with the Cy3-labeled anti-FLAG antibody at 1:200 dilution at 4°C for 1 hr and washed twice in ice-cold PBS. Cells were then incubated in the culture medium at 37 for the indicated periods to allow internalization followed by fixation in methanol at –20°C.

For quantitative comparison, cells expressing AE1 proteins, as judged by EGFP signal expression, were classified into 5 stages according to the localization of the signals of Cy3-labeled AE1: stage 1, with abundant cell-surface signals and without cytoplasmic signals; stage 2, with cell-surface signals and some intracellular spots; stage 3, with predominant intracellular signals; stage 4, with intracellular signals only; and stage 5, with no signals. For each AE1, 200 cells were categorized and the percentage at each stage was determined. Data are given as the means ± S.D. from three independent experiments.

The internalization of transferrin was chased as reported previously [27] by using aminomethylcoumarin acetate-conjugated streptavidin (Dako) and biotinylated holo-transferrin (Sigma).

Statistical analysis: The statistical significance of differences in the data was assessed with the paired Student's *t*-test.

RESULTS

Cell-surface expression of EGFP-mAE1Flag and other mutants in K562 cells: We first analyzed the expression of EGFP-mAE1Flag and its Y72F and bNt75 mutants, neither of which have the YXXΦ-type sequence in their N-terminal stretches, in K562 cells (Fig. 2). The EGFP signals were predominantly observed at the periphery of the cells, and no obvious difference in localization was found between EGFP-mAE1Flag and other mutants, indicating that these AE1 mutants were properly targeted to the plasma membrane in K562 cells (Fig. 2A). The extracellular FLAG tag of EGFP-mAE1Flag was specifically labeled with the Cy3-labeled anti-FLAG antibody (Fig. 2B). In immunoblotting, these AE1 polypeptides were detected as broad bands with apparent molecular masses of 140,000–150,000 corresponding to the expected sizes of glycosylated murine AE1 tagged with EGFP and their expression levels were almost identical (Fig. 2C).

Several independent experiments of cell surface biotinylation and densitometric scanning of immunoblotting signals showed that the abundance of cell surface AE1 polypeptides relative to the total amount for the Y72F mutant (33% ± 2%, n=4) was significantly higher than that for EGFP-mAE1Flag (22% ± 1%, n=4) (Fig. 2D). The bNt75 mutant also had a value higher than that of EGFP-mAE1Flag but the difference was not statistically significant. Deglycosylation with endoglycosidase H (endo H) and peptide-*N*-glycosidase F (PNGase) demonstrated that EGFP-mAE1Flag in the cell-surface fraction and in the intracellular fraction contained polypeptides with either endo H-resistant or endo H-sensitive *N*-glycans. The population of EGFP-mAE1Flag with endo H-sensitive *N*-glycan was significantly ($P<0.01$) less abundant in the cell surface fraction (32% ± 2%, n=4) compared to that in the intracellular fraction (39% ± 2%, n=4). A similar difference was also observed in the cells expressing Y72F and bNt75 mutants, although the differences were not statistically significant.

These data indicate that the presence or absence of the Y72VEL sequence may affect the steady-state levels of these AE1 proteins on the cell surface. The data also showed that at least 30% of cell surface AE1 was transported to the plasma membrane without processing of *N*-glycan in the Golgi apparatus, suggesting that the N-terminal YXXΦ motif participated in the internalization and recycling of EGFP-mAE1Flag.

YXXΦ motif-mediated endocytosis of EGFP-mAE1Flag: To test this hypothesis, cell surface AE1 mutants were labeled with the Cy3- anti-FLAG antibody and chased for their changes in localization. Before incubation, Cy3-labeled EGFP-mAE1Flag was distributed at the cell surface with a granular appearance (Fig. 3A). Incubation at 37°C

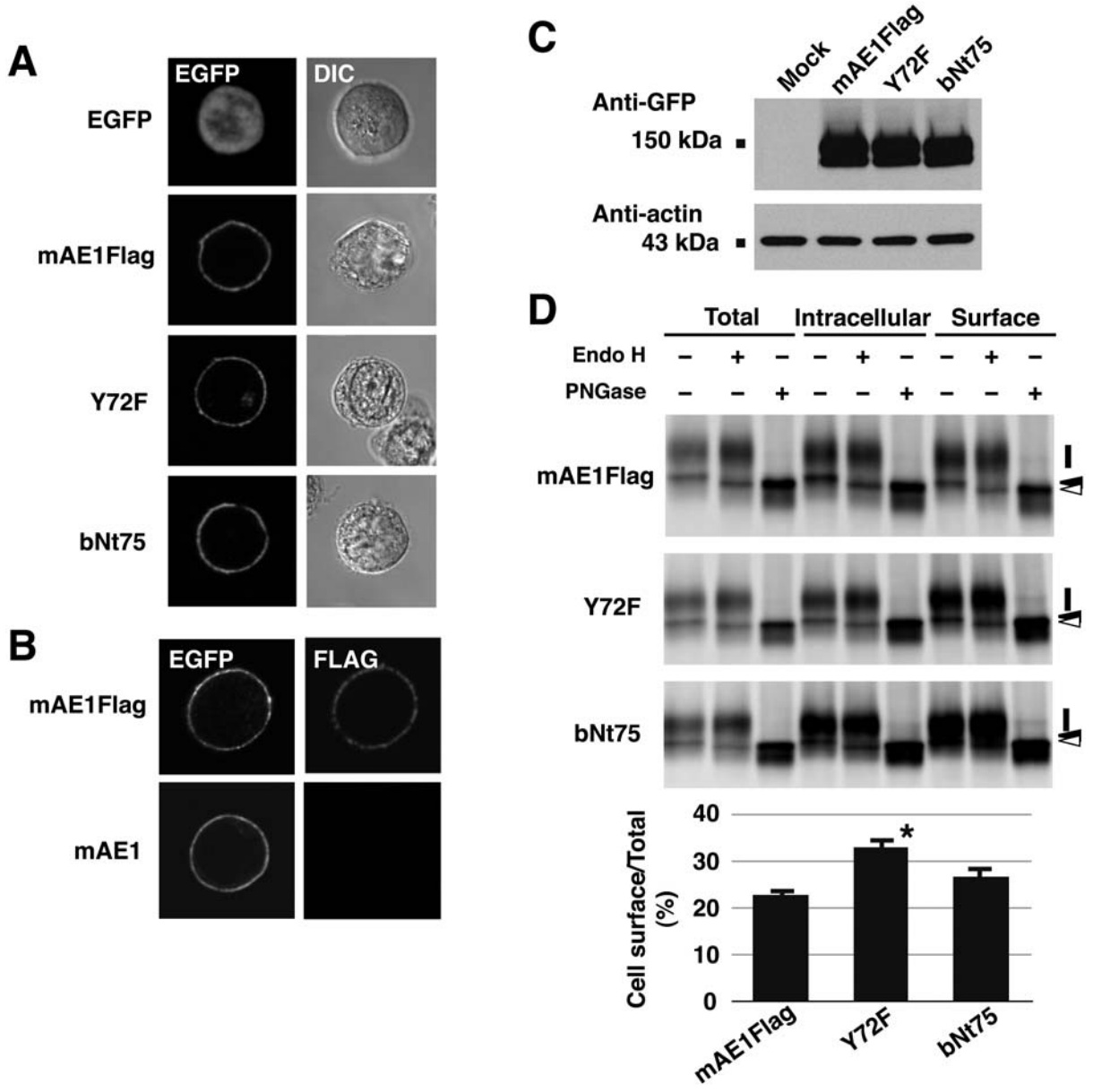

Fig. 2. Expression of EGFP-mAE1Flag and its Y72F and bNt75 mutants in K562 cells. (A) EGFP fluorescence (*EGFP*) and the corresponding differential interference contrast (*DIC*) images of K562 cells stably expressing EGFP, EGFP-mAE1Flag (*mAE1Flag*), Y72F and bNt75 mutants are shown. (B) When reacted with the Cy3-anti-FLAG antibody, Cy3 signals for FLAG tag (*FLAG*) comparable with those of EGFP were found in the cells expressing EGFP-mAE1Flag (*mAE1Flag*) but not in the cells expressing EGFP-tagged murine AE1 without FLAG tag (*mAE1*). (C) Expression levels of AE1 mutants were compared with those of β-actin in total detergent extracts of K562 cells by immunoblotting the same blot with anti-GFP (*Anti-GFP*) and anti-β-actin (*Anti-actin*) antibodies. Lane *Mock* includes the lysate from cells expressing EGFP. Migrating positions of the markers are indicated in kDa. (D) Cell-surface biotinylation and deglycosylation of AE1 proteins in K562 cells. Cell-surface proteins were biotinylated with NHS-SS-biotin. The obtained total cell lysate (*Total*) was separated into bound (*Cell surface*) and unbound (*Intracellular*) fractions by using NutrAvidin beads. The proteins were deglycosylated with endo H (*Endo H*) or PNGase (*PNGase*), followed by the detection of AE1 mutants in immunoblotting, as described above. Bars and closed and open arrowheads indicate migrating positions of AE1 polypeptides of which the *N*-glycan is endo H-resistant, endo H-sensitive, and deglycosylated, respectively. The abundance of cell-surface AE1 polypeptides relative to the total content determined by densitometric scanning of the immunoblots is shown at the bottom. Data represent the means ± S.D. (n=4). Asterisk indicates a significant difference relative to the value of EGFP-mAE1Flag (P<0.01).

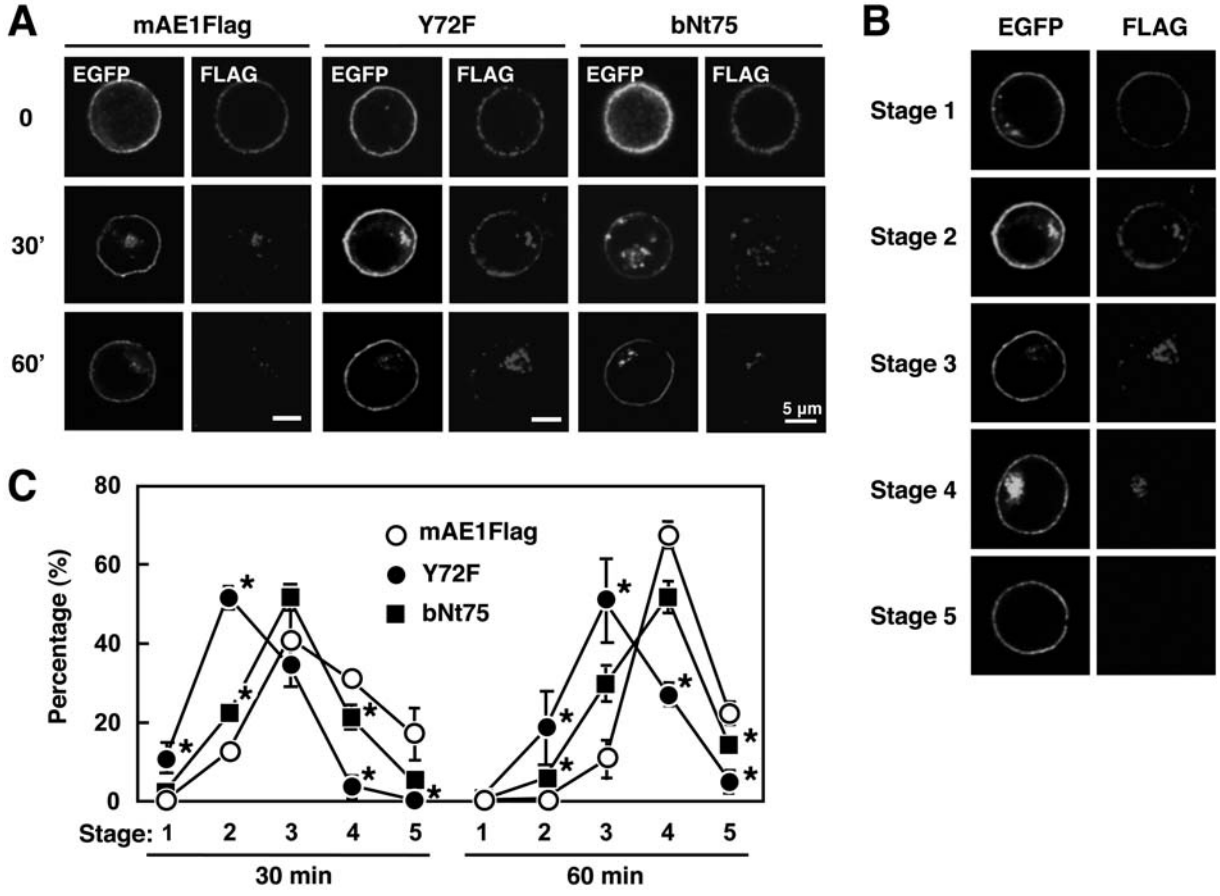

Fig. 3. Internalization of EGFP-mAE1Flag and the N-terminal mutants in K562 cells. (A) The EGFP-mAE1Flag (*mAE1Flag*), Y72F (*Y72F*) and bNt75 (*bNt75*) mutants expressed at the surface of K562 cells were labeled with the Cy3-anti-FLAG antibody at 4°C for 1 hr. After incubation at 37°C for 0, 30 or 60 min, cells were fixed and examined for EGFP and Cy3 signals. Bars, 5 μm. (B) Cells were divided into five stages (*stages 1—5*) according to the localization of the Cy3 signals as described in Materials and Methods. EGFP (*EGFP*) and Cy3 (*FLAG*) signals of the typical cell at each stage are shown. (C) Internalization statuses of EGFP-mAE1Flag (*open circles*), Y72F (*closed circles*) and bNt75 (*closed squares*) after incubation for 30 or 60 min at 37°C. Two-hundred cells possessing EGFP signals were grouped into 5 stages according to the localization of the Cy3 signals as described above, and the percentages of the cells at each stage were determined. Data represent the means ± S.D. from three independent experiments. Asterisks indicate a significant difference relative to the value of EGFP-mAE1Flag (P<0.01, n=3).

caused the rapid internalization of Cy3 signals in 10 min. Cell-surface signals disappeared after 30 min of incubation. This disappearance resulted in the juxtanuclear formation of a cluster of vesicular structures, followed by a remarkable reduction in signals at 60 min. The presence of EGFP signals coincident with Cy3 signals confirmed that the Cy3 signal represented that of the internalized AE1 polypeptides. The bNt75 mutant showed similar behavior to that of EGFP-mAE1Flag. In contrast to these AE1 polypeptides, abundant signals for the Y72F mutant were retained at the cell surface at 30 min and in the cytosol at 60 min of incubation, which suggested delayed internalization of this mutant.

To determine precisely the difference in internalization efficiency of these AE1 polypeptides, we grouped the cells expressing each AE1 into 5 distinguishable stages of internalization (Fig. 3B). The Cy3 signals were internalized in the vast majority of the cells expressing EGFP-mAE1Flag (stages 3–5, 88% ± 7%, n=3) at 30 min, when only a small population (stage 2, 12% ± 1%, n=3) had signals with reduced intensities at the plasma membrane (Fig. 3C). In contrast, the Cy3 signals remained at the cell surface in more than 60% of the cells possessing the Y72F mutant (stages 1 and 2, 63% ± 4%, n=3). A further 30-min incubation caused the remarkable reduction (stages 3 and 4, 67% ± 5%, n=3) or disappearance (stage 5, 22% ± 2%, n=3) of signals for EGFP-mAE1Flag, whereas the signals were still observed at the cell surface in 67% ± 10% of cells (stages 2 and 3, n=3) expressing the Y72F mutant. Cells expressing the bNt75 mutant showed profiles that were basically the same as those of cells expressing EGFP-mAE1Flag, although the population at stages 1–3 was larger than that of the cells expressing EGFP-mAE1Flag.

We then compared the internalization of AE1 in K562 cells with that of the transferrin receptor (TfR), a typical

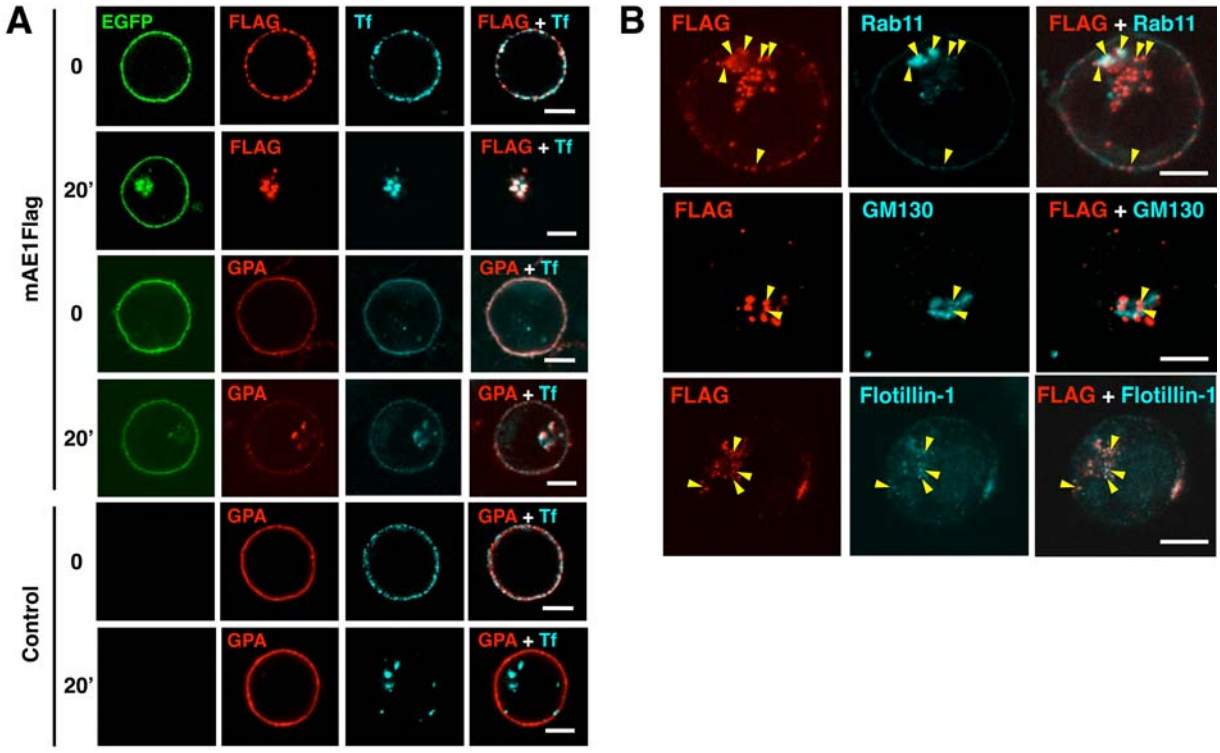

Fig. 4. Internalization of EGFP-mAE1Flag, GPA, and transferrin in K562 cells. (A) Cell surface EGFP-mAE1Flag, GPA, and TfR were labeled with appropriate antibodies (*FLAG* and *GPA*) or fluorescence-labeled transferrin (*Tf*). After incubation at 37°C for 0 or 20 min, cells were fixed and analyzed for fluorescent signals. Merged images (*FLAG + TF* and *GPA + Tf*) are also shown. (B) Intracellular distributions of EGFP-mAE1Flag (*FLAG*) internalized with incubation for 20 min were compared with those of several organelle markers, including Rab11 (*Rab11*), GM130 (*GM130*), and flotillin-1 (*Flotillin-1*). Colocalization of Cy3 and organelle markers are indicated by arrowheads in merged images (*FLAG + Marker*). Bars, 5 μm.

cargo for clathrin-mediated endocytosis with the YXXΦ motif [26], using fluorescence-labeled transferrin. EGFP-mAE1Flag and transferrin showed comparable localization with granular features at the cell surface before incubation. At 20 min after incubation at 37°C, signals for endocytosed transferrin were coincident with those of internalized EGFP-mAE1Flag (Fig. 4A). This phenomenon was also observed for Y72F and bNt75 mutants (data not shown). Glycophorin A (GPA), an AE1 chaperone-like sialoglycoprotein with a single membrane span in erythroid cells [28], also showed the rapid internalization in cells expressing EGFP-mAE1Flag. The signals for internalized GPA were coincident with those of transferrin and EGFP. In contrast, GPA exhibited no remarkable internalization after a 20-min incubation in control K562 cells without transfectants.

Immunofluorescent Cy3 signals of internalized EGFP-mAE1Flag were close to and partially coincident with those of several distinct organelle markers, including Rab11 for the endocytic recycling compartment and GM130 for the Golgi (Fig. 4B). Several Cy3 signals also showed colocalization with the immunofluorescent signals of flotillin-1 that has been shown to reside in some unidentified endocytic intermediates [10]. This finding indicates that EGFP-mAE1Flag resided in some compartments at juxtanuclear areas adjacent to the organelles involved in endocytic recycling.

Taken together, these results demonstrate that EGFP-mAE1Flag was internalized principally through the clathrin-mediated endocytic pathway that was facilitated by the YXXΦ (Y72VEL) sequence and that the endocytosis of EGFP-mAE1Flag was associated with GPA internalization in K562 cells. The data also suggest that the bNt75 mutant had some alternative signal(s) that enhanced the endocytosis.

Identification of the Y7EDQL sequence as an alternative signal for AE1 endocytosis: Because the EGFP-mAE1Flag and bNt75 mutants transiently expressed in HEK293 cells showed rapid internalization, whereas the Y72F mutant had remarkably reduced endocytosis (as observed in K562 cells; Fig. 5A for the bNt75 mutant), we analyzed the effects of further mutations (Fig. 1) on endocytosis in HEK293 cells to determine the element that allowed the bNt75 mutant to be endocytosed.

Several deletion mutants, i.e., bNtΔ15, bNtΔ29, and bNtΔ50, exhibited cell-surface retention even after 60 min of incubation (Fig. 5B). This finding indicates that the removal of N-terminal 15 amino acids was enough to reduce the endocytosis of bNt75 EGFP-mAE1Flag. This region

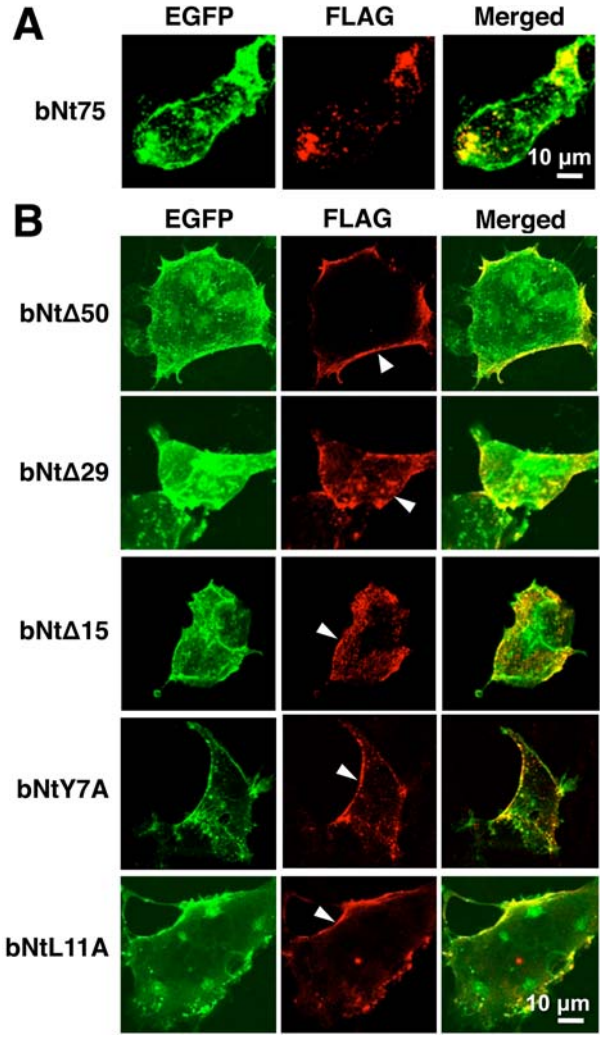

Fig. 5. Internalization of various AE1 mutants in HEK293 cells. (A) HEK293 cells transfected with bNt75 mutant were labeled with the Cy3-anti-FLAG antibody and analyzed for Cy3 signal distributions after incubation for 60 min at 37°C. (B) HEK293 cells were transfected with several deletion mutants lacking the N-terminal 15 (*bNtΔ15*), 29 (*bNtΔ29*) or 50 (*bNtΔ50*) amino acids and Y7A (*bNtY7A*) and L11A (*bNtL11A*) substitution mutants. Labeling of FLAG tags and incubation were carried out as described above. All of these mutants retained the Cy3-labeled AE1 polypeptides at the cell surface, as indicated by arrowheads. Bars, 10 μm.

contains the Y7EDQL sequence, which was likely responsible for endocytosis because the YXXΦ motif has some flexibility in its recognition [22, 26]. In fact, the Cy3 signals of the bNtY7A and bNtL11A mutants remained at the surface of the transfected cells after a 60-min incubation. This observation demonstrates that Y7 and L11 were critical residues for the internalization of bNt75 EGFP-mAE1Flag (Fig. 5B). Thus, the Y7EDQL sequence in the N-terminal stretch of bovine AE1 appears to compose a noncanonical YXXXΦ-type internalization signal.

DISCUSSION

Because the N-terminal YVEL sequence is well conserved among species, the clathrin-dependent and YXXΦ motif-mediated endocytosis demonstrated in the present study appears to be a fundamental characteristic of erythroid AE1 in various mammals. The precise mechanism for the assembly of erythroid AE1 into the organized structure of the membrane skeleton, that is seen in the mature erythrocyte [16], is undefined yet. However, recent studies indicate that the formation of the membrane skeletal network, including the assembly of AE1 itself, occurs progressively during erythroid cell maturation [5, 15]. Cell-surface expression of AE1 and GPA is found in early-stage erythroblasts and remarkably increased in late-stage erythroblasts in accordance with the increase in expression of the membrane skeletal proteins [5]. In contrast, TfR shows little change in the surface expression between early- and late-stage erythroblasts [5] and plays an essential role in iron delivery by repeating the endocytosis and recycling back to the cell surface [20]. Therefore, our present findings suggest that the clathrin-dependent internalization of AE1 in association with GPA likely occurs during erythroid cell maturation.

Physiological roles of AE1 endocytosis may include *N*-glycan maturation and association with ankyrin during recycling to the Golgi, as previously suggested in chicken erythroid cells [27]. Partial colocalization of the internalized EGFP-mAE1Flag with the endocytic recycling compartment and the Golgi apparatus (Fig. 4) and the presence of endo H-sensitive *N*-glycan in the cell-surface fraction of AE1 (Fig. 2) support this assumption. The endocytosis followed by recycling back to the plasma membrane is also compatible with our previous finding that approximately 90% of the wild-type bovine AE1 pulse-labeled in the K562 cells remained intact after 24 hr and about 10% of that protein was detected at the plasma membrane throughout the chase period [12]. However, we could not see the recycling of internalized AE1 to the cell surface and the Cy3 signals were all diminished after a 60-min incubation (Fig. 3). These are possibly due to dissociation of the Cy3-anti-FLAG antibody from the AE1 under reduced pH condition in endocytosed vesicles and subsequent degradation of the antibody. Thus the recycling of AE1 to the plasma membrane remains to be proven in mammalian erythroid precursors.

The endocytosis of erythroid AE1 reported here is consistent with the internalization of several variants of chicken AE1, possibly through clathrin-mediated pathways [9], although the involvement of YXXΦ motifs corresponding to the Y72VEL sequence is unknown. However, previous studies have shown that the Y47VEL sequence is involved in basolateral sorting and Golgi recycling [2, 3] via a caveolin-dependent and clathrin-independent pathway [8] of AE1-4, a kidney variant of chicken AE1, in MDCK cells. Because AE1-4 and erythroid variants (AE1-1 and -2) of chicken AE1 share the Y47VEL sequence [6] (Fig. 1), it is

suspected that this YXXΦ motif directs the endocytosis of erythroid and kidney AE1 through a different pathway.

In mammals, however, the internalization of erythroid and kidney AE1 probably uses the YXXΦ motifs at distinct positions. Our present data show that the N-terminal YXXΦ motif facilitates the internalization of AE1 in K562 and HEK293 cells (Figs. 3 and 5). This internalization is independent of the conserved C-terminal YXXΦ sequence that is essential in the basolateral sorting of human kidney AE1 [7, 24]. Basolateral distribution of human kidney AE1 also requires cooperative control by the phosphorylation status of 2 tyrosine residues in Y904DEV at the C-terminus and Y359KGL in the N-terminal cytoplasmic domain [29]. Structurally, the conformation of the cytoplasmic domain of human kidney AE1 is less stable and more open than that of erythroid AE1 [18, 30]. This situation likely enables the cooperative interaction of the N-terminal domain with the C-terminus in kidney AE1, whereas it may generate a role for the erythroid N-terminal YXXΦ motif that is independent of the C-terminal motif as shown in the present study.

This assumption does not rule out the possibility that the C-terminal YXXΦ motif itself acts as an endocytic signal because AE1 polypeptides are still internalized in the absence of the N-terminal motif (Figs. 3 and 5). Moreover, it should be noted that a small population of internalized EGFP-mAE1Flag colocalizes with flotillin-1 (Fig. 4). The previous study has shown that flotillin-1 is associated with endocytosis via a pathway that is independent of both clathrin and caveolin in HeLa and COS-7 cells [10]. Therefore, our data suggest that several distinct pathways are involved in the endocytosis of AE1, although there are some other possibilities including the presence of flotillin-1 in clathrin-coated vesicles in K562 cells and/or the mixing of protein constituents by the fusion of the vesicles endocytosed via different pathways.

The present study also demonstrates that a sequence Y7EDQL, representing YXXXΦ in the most N-terminal region of bovine AE1, can mediate the endocytosis of AE1. The YXXΦ motif present in cargo molecules binds to a specifc region in the μ2 subunit of the sorting adaptor AP2 and the spacing between the Y and Φ residues is crucial for the interaction with the μ2 subunit [17]. However, evidences have demonstrated that there is also flexibility in recognition of the YXXΦ motif by the AP2 clathrin adaptor complex. For instance, the ionotropic P2X4 ATP receptor that rapidly cycles off the plasma membrane uses a YXXGΦ signal for its endocytosis. The YXXGΦ motif recognizes the same hydrophobic pockets in the μ2 subunit but accommodates the extra glycine residue by altering the backbone configuration of the peptide [22]. Several other YXXXΦ motifs reported include the sequences YSGTI in thromboxane A2 receptor β [19] and YKSTF in the adherens junction protein shrew-1 [13]. Our finding is compatible with these previous studies and emphasizes that some variation in number and composition between Y and Φ is permitted in YXXΦ motif-μ2 interactions. However, it is likely that the YXXΦ and YXXXΦ motifs are different in their abilities for binding with AP2 because distinct internalization profiles were observed for EGFP-mAE1Flag and the bNt75 mutant (Fig. 3).

In conclusion, our present study demonstrates that a conserved YXXΦ motif and a noncanonical YXXXΦ motif in the N-terminal stretch facilitates the clathrin-mediated internalization of erythroid AE1. These observations suggest an endocytic recycling process of AE1 during erythroid cell maturation.

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