Tetraspanin CD63 Promotes Targetion and Lysosomal Proteolysis of Membrance-Type 1 Matrix Metalloproteinase

Tetraspanin CD63 Promotes Targeting and Lysosomal Proteolysis of Membrane-Type 1 Matrix Metalloproteinase

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SUMMARY

Membrane-type 1 matrix metalloproteinase (MT1-MMP) is known to be internalized from cell surface, however, the fate of internalized MT1-MMP is still unknown. Here we demonstrate that at least a part of internalized MT1-MMP is targeted for lysosomal proteolysis. Treatment with an inhibitor of lysosomal proteinases chloroquine suppressed degradation of internalized MT1-MMP, and induced accumulation of MT1-MMP in CD63-positive lysosomes. Ectopic expression of CD63 accelerated degradation of MT1-MMP, which was blocked by chloroquine. MT1-MMP and CD63 were shown to form a complex through hemopexin-like domain of MT1-MMP and N-terminal region of CD63, and thus accelerated degradation of MT1-MMP was not observed with mutants lacking these domains. CD63 mutant lacking lysosomal targeting motif was unable to promote MT1-MMP degradation. These results suggest that CD63 regulates MT1-MMP by targeting to lysosomes.

Keywords: CD63; internalization; lysosomes; MMP; MT1-MMP; proteolysis; tetraspanins

Matrix metalloproteinases (MMP) are a family of Zn^{2+}-dependent enzymes that are essential for extracellular matrix (ECM) turnover in normal and pathological conditions. MMP anchored to plasma membrane are subgrouped into membrane-type MMP (MT-MMP) [1-5]. Of all the MT-MMPs, MT1-MMP is believed to be a crucial for the invasion of malignant tumors [1, 5, 6]. MT1-MMP activity is regulated by several distinct mechanisms. To express on cell surface, proMT1-MMP requires for being processed by proprotein convertases [7]. The enzymatic activity on the cell surface can be inhibited by the tissue inhibitors of metalloproteinases (TIMP)-2, -3, and -4 but not by TIMP-1. After MMP-2 activation, a part of MT1-MMP is down-regulated through processing by activated MMP-2 and autocatalytically [8]. Resent studies have revealed that the cell-surface MT1-MMP is regulated by internalization [9, 10]. However, it is largely unknown whether MT1-MMP is degraded at endosomes/lysosomes, or whether internalized MT1-MMP is recycled back to the plasma membrane.

Tetraspanins of integral membrane proteins with four predicted transmembrane domains comprises a large group of ubiquitously expressed proteins that function in diverse contexts such as T- and B-cell activation, and cell migration and proliferation [11]. CD63 is a member of tetraspanins and a well-established component of the late endosomal and lysosomal membranes [12]. CD63 is highly expressed in primary melanoma but weaker or absent in metastatic melanomas [13, 14]. Ectopic expression of CD63 in melanoma cells suppresses their metastatic

potential in animal model system [15] . In the present study, we analyzed the fate of internalized MT1-MMP and the effect of CD63 on the stability of MT1-MMP and demonstrated that CD63 plays at least in part a role in promoting lysosomal proteolysis of MT1-MMP.

MATERIALS AND METHODS

Cell Culture and Materials. MDCK cells and HT1080 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). HeLa cells and 293-EBNA cells (Invitrogen) were cultured in DMEM supplemented with 10% FBS. MMP inhibitor, BB94 was prepared as described previously [16]. Antibodies used were: anti-actin and anti-FLAG M2 mouse monoclonal antibodies (Sigma-Aldrich); an anti-GFP rabbit polyclonal antibody (Santa Cruz Biotechnology); anti-CD63 mouse monoclonal and anti-MT1-MMP rabbit polyclonal antibodies (Chemicon); horseradish peroxidase (HRP) conjugated secondary antibodies and streptavidin (Amersham Pharmacia Biothech).

Expression Plasmids. pSG5-MMP-2, FLAG epitope tagged-MT1-MMP (MT1F), the catalytic domain-deleted mutant (ΔCat), the hemopexin-like domain-deleted mutant (ΔPex) and the cytoplasmic tail-deleted mutant (ΔCP) of MT1F were constructed as described previously [10]. pRK-GFP was provided from Dr. K.M. Yamada (National Institutes of Health, Bethesda, MD). The puromycin resistance plasmid pHA262pur was kindly provided by Dr. Hein te Riele (Netherlands

Cancer Institute, Amsterdam, The Netherlands). A cDNA enconding CD63 (GenBank accession number BC002349) was obtained by RT-PCR with human placenta cDNA and was cloned into the pRK-GFP expression vector. pRK-GFP-CD63 was used as a template for PCR-based modification to generate the deletion mutants for the small extracellular loop (Met^{1}-Ser^{51})-deleted mutant (ΔSEL), the large extracxellular loop (Val^{106}-Lys^{186})-deleted mutant (ΔLEL), the cytoplasmic domain-deleted mutant (ΔC12; Leu^{227}-Met^{238}) of GFP-CD63.

Transfection. Transient transfections were performed by either calcium phosphate methods or using Trans IT-LT1® (PanVera) according to the manufacture's instructions. To obtain MDCK cells stably expressing GFP-CD63, MDCK cells were co-transfected with pRK-GFP-CD63 and pHA262pur plasmids and selected in medium containing puromycin.

Immunoprecipitations and Immunoblottings. The cells were washed with ice-cold phosphate buffered saline (PBS), homogenized in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1% TritonX-100. The cell lysates were used for immunoprecipitation with anti-FLAG-M2 antibody. The immunoprecipitates were subjected to immunoblotting using indicated antibodies.

Gelatin Zymography. Gelatin zymography was performed with SDS-polyacrylamide gel containing 0.1% gelatin as described previously [3]. Gelatinolytic activity was visualized as a negative staining with Coomassie Brilliant Blue.

Cell-Surface Biotinylation. Transfected HeLa cells were washed with ice-cold PBS, incubated with 0.5 mg/ml sulfo-NHS-biotin (Pierce) in PBS at 4°C for 30 min, and then further incubated with 25 mM lysine. The cells were cultured for 3 h in complete medium with or without chloroquine (116 μM), homogenized and immunoprecipitated with anti-FLAG M2 antibody. The immunoprecipitates were analyzed by immunoblotting using streptavidine-HRP or anti-FLAG antibody.

Immunofluorescence Staining. For cell-surface staining, cells were incubated for 30 min at 4°C with anti-FLAG M2 antibody. After washing, cells were incubated in complete media with BB94 (1 μM) at 37°C for indicated period. The cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with (permeabilized) or without (non-permeabilized) 4% paraformaldehyde/0.5% Triton X-100 for 5min, and then visualized with Cy3- or Alexa™488-conjugated goat anti-mouse IgG (Molecular Probes) using confocal laser microscopy (Carl Zeiss). For CD63 staining, cells were fixed with 4% paraformaldehyde for 20 min, treated for 30 min with 0.27% NH_4Cl/0.38% glycin in PBS, and permeabilized for 30 min with 0.05% saponin in PBS.

RESULTS

Internalized MT1-MMP is localized at CD63-positive lysosomes. Internalization of

FLAG-tagged MT1-MMP (MT1F) in HeLa cells was examined by fluorescence immunostaining. MT1F was localized at the periphery of permeabilized and non- permeabilized cells at 0 time (Fig. 1A). While the signal became weaker at 30 min and undetectable at 3 h after labeling in non-permeabilized cells, MT1F was detected in cytoplasm as dot-like spots at 3 h after labeling in permeabilized cells. Next, to examine whether MT1F is subjected to lysosomal proteolysis, the effect of a lysosomal proteases inhibitor chloroquine was studied (Fig. 1B). MT1F was localized mainly in the cytoplasm in un-treated cells, however, chloroquine treatment induced accumulation of MT1F and co-localization with CD63 as dot-like spots. Suppression of degradation of surface-labeled MT1-MMP by chloroquine was confirmed by western blotting (Fig. 1C). These results indicate that a part of MT1-MMP internalized from cell surface is transported and degraded at CD63-positive lysosomes.

CD63 down-regulates MT1-MMP. The effect of CD63 on MT1-MMP expression was examined. Co-expression of MT1F with MMP-2 induced processing of MMP-2 from 68-kDa latent form to 64-kDa intermediate and 62-kDa active forms as shown by gelatin zymography (Fig. 2A). Transfection of CD63 plasmid down-regulated MMP-2 processing by dose dependent manner. Expression level of MT1-MMP was drastically reduced by CD63 expression as demonstrated by western blotting (Fig. 2B), which was clearly restored by the treatment of cells with chloroquine. MMP inhibitor BB94 had no effect. These results suggest that CD63

expression reduces the level of cell-surface MT1-MMP and promotes its lysosomal proteolysis. To analyze the mechanism of CD63-dependent proteolysis of MT1-MMP, cell-surface MT1-MMP on CD63-expressing cells was pulse-labeled with the antibody, and the internalization of MT1-MMP was monitored. Internalized MT1-MMP accumulated and co-localized with CD63 in lysosomes (Fig. 2C). These results may suggest that reduction of cell surface MT1-MMP by CD63 expression is due to promoted internalization and degradation at lysosome.

Interaction between CD63 and MT1-MMP. To analyze the interaction between MT1-MMP and CD63, immunoprecipitation was performed. As shown in Fig. 3B, CD63 was co-precipitated with MT1F. CD63 mutants lacking the C-terminal cytoplasmic tail or the C-terminal large extracellular loop (LEL) (ΔLEL) was co-precipitated with MT1-MMP, while mutants lacking the N-terminal small extracellular loop (SEL) (ΔSEL) of CD63 failed. In addition, although WT-CD63 reduced the level of MT1-MMP, the ΔC12, ΔSEL, and ΔLEL mutants of CD63 did not. The MT1-MMP mutant lacking hemopexin-like domain (ΔPex) was not co-precipitated with CD63, whereas other mutants of MT1-MMP did (Fig. 3D). Consequently, co-expression of CD63 reduced the level of MT1F but not that of MT1FΔPex (Fig. 3E). These results suggest that N-terminus of CD63 and hemopexin-like domain of MT1-MMP are necessary for the association, but not sufficient to promote lysosomal proteolysis of MT1-MMP.

Role of cytoplasmic tails of MT1-MMP and CD63. The C-terminal cytoplasmic tail of CD63 contains the lysosomal targeting motif GYEYM, which interacts with the μ2 subunit of adaptor protein (AP)-2 adaptor and the μ3 subunit of AP-3 [17, 18]. In fact, the deficiency of AP-3 results in increased cell-surface expression of CD63 due to lack of its lysosomal trafficking [17]. The role of CD63 C-terminal cytoplasmic tail in promotion of MT1-MMP lysosomal proteolysis was studied. MT1F was localized at cell periphery in the cells expressing MT1F alone, and cell-surface staining of MT1F was significantly reduced in cells co-expressing GFP-CD63 (Fig. 4A). CD63 accumulated at lysosomes, however, CD63ΔC12 was mainly distributed in cytoplasm and cell periphery. Cell-surface localization of MT1F was not significantly affected by CD63ΔC12 expression. Figure 4B showed the relative levels of cell-surface MT1-MMP in cells co-expressing CD63 or their mutants. CD63 expression markedly reduced cell-surface expression of MT1-MMP, but MT1FΔPex was not significantly reduced by CD63 expression. Cell-surface expression of MT1FΔCP was faintly down-regulated by CD63 expression. In addition, CD63ΔC12 expression did not attenuate cell-surface expression of MT1F. Hence, CD63 expression apparently down-regulated MMP-2 activation mediated by MT1F, but that by MT1FΔCP was affected only faintly by CD63 expression (Fig. 4C).

DISCUSSION

In the present study we demonstrated for the first time that a part of MT1-MMP internalized from cell surface is transported to CD63-positive lysosomes and degraded there. Ectopic expression of CD63 promoted lysosomal proteolysis of MT1-MMP (Fig. 2B). The direct interaction between MT1-MMP and CD63 was shown to be mediated through the hemopexin-like domain of MT1-MMP and N-terminal region of CD63 (Fig. 3). Thus, MT1-MMP level was not altered by the expression of CD63ΔSEL, and MT1FΔPex by CD63 (Fig. 4). Targeting of the complex to lysosome is mediated by lysosomal targeting motif of CD63 (GYEYM) which is located at the C-terminal tail, and thus CD63ΔC12 was unable to stimulate lysosomal proteolysis of MT1-MMP (Fig. 3B).

The importance of cytoplsmic tail of MT1-MMP for the internalization from cell surfac was demonstrated by Uekita et al. [10] and in this study (Fig. 4). The cytoplasmic tails of MT1-MMP and CD63 interact with the μ2 subunit of AP-2 adaptor. Thus, the interaction between MT1-MMP and CD63 may promote their internalization. The facts that expression of CD63 is ubiquitous and internalized MT1-MMP is co-localized with endogenous CD63 at lysosomes suggest that CD63 is one of regulators of MT1-MMP. However, the mechanism of CD63-mediated internalization of MT1-MMP still remained examined. Further study is required to clarify whether the association of CD63/MT1-MMP complex with integrin $\alpha_3\beta_1$, CD81, phosphatidylinositol 4 kinase [19], and

other proteins such as CD44 [20] and claudins [21] can regulate MT1-MMP enzymatic activity and dynamic turnover of MT1-MMP at cell periphery.

In conclusion, we have shown that CD63 is involved in the internalization of MT1-MMP from cell-surface, lysosomal targeting and proteolysis of MT1-MMP. Understanding the mechanism by which MT1-MMP is internalized from cell surface, transported to lysosomes, and presumably recycled back to plasma membrane might provide a step to regulate MT1-MMP activity which is closely associated with the invasiveness and metastasis of malignant tumor cells.

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FIGURE LEGENDS

FIG. 1. Localization of internalized MT1-MMP. (A) HeLa cells were transfected with MT1F, and

incubated with anti-FLAG antibody for 30 min at 4 °C. After washing, the cells were cultured in complete media for 0, 30 min, or 3 h, and then performed immunofluorescence staining under either permeabilized (+TX100) or non-permeabilized (-TX100) conditions as described in Materials and Methods. (B) HeLa cells were transfected with MT1F, and cultured in the presence (+) or absence (-) of chloroquine (CHQ; 58 μM) for 6h. The cells were fixed under permeabilized condition, and then stained with anti-CD63 and anti-MT1-MMP antibodies. Bar, 20 μm. (C) Cell-surface biotinylation and immunoprecipitation were carried out as described in Materials and Methods. Cell-surface MT1F was detected by streptavidine-HRP (Blot: SA-HRP), and total MT1F was by anti-FLAG antibody (Blot: FLAG). The whole-cell lysates (WCL) were immunoblotted with anti-actin (Blot: Actin) antibody.

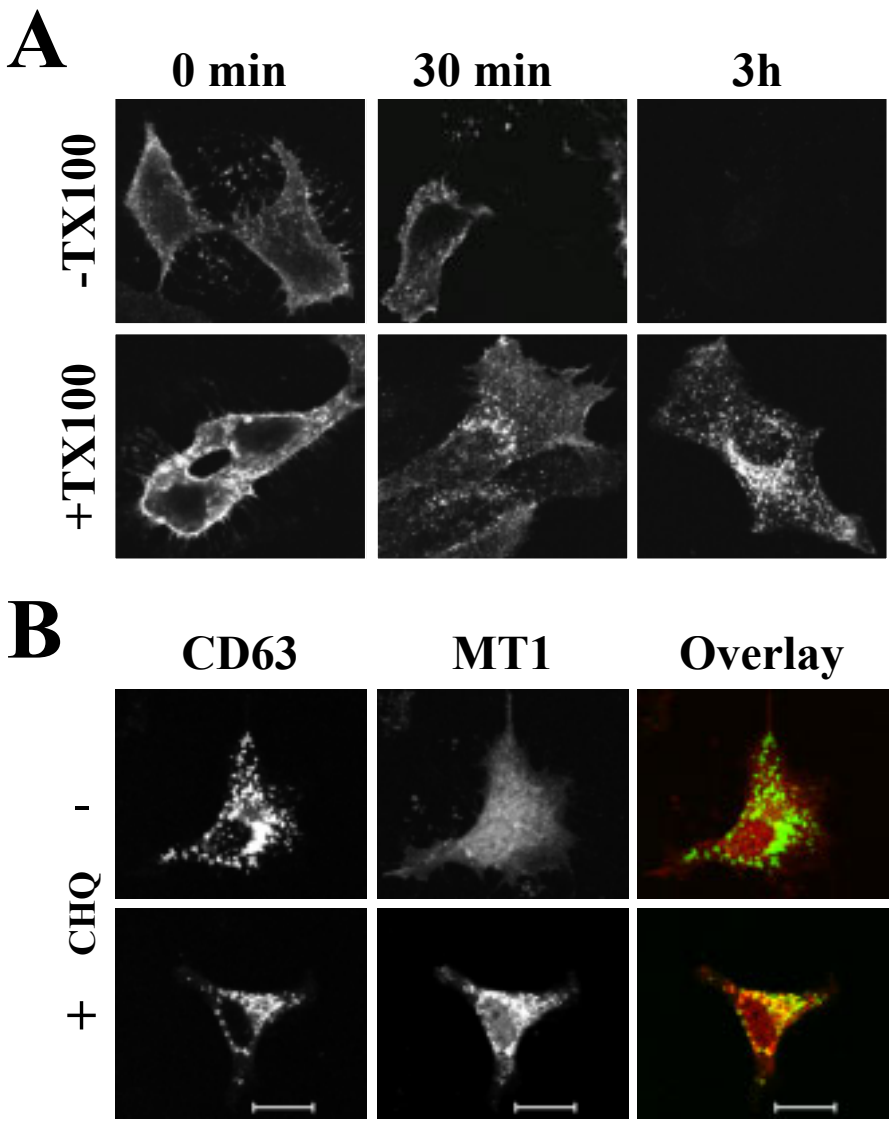
FIG. 2. CD63 down-regulates MT1-MMP. (A) 293-EBNA cells were co-transfected with MMP-2, MT1F and 50 ng, 200 ng, or 1 μg of GFP-CD63 plasmids. The conditioned media were analyzed by gelatin zymography. (B) 293-EBNA cells were co-transfected with MT1F and either GFP or GFP-CD63 plasmids, and treated with DMSO, BB94, chroloquine (CHQ) overnight. The cells were homogenized, and immunoblotted with anti-FLAG (Blot: FLAG) or anti-GFP (Blot: GFP) antibody. (C) MDCK cells stably expressing GFP-CD63 were transiently transfected with MT1F, incubated with anti-FLAG antibody conjugated with Alexa™546 for 30 min, and cultured further

30 min or 6 h in the presence of BB94 with or without chloroquine (CHQ). The cells were fixed with 4% paraformaldehyde. Bar, 20 μm.

FIG. 3. Interaction between CD63 and MT1-MMP. (A) CD63 composes with four transmembrane domains (TM1 to TM4), and two extracellular loop, SEL and LEL. 293-EBNA cells were co-transfected with MT1F and GFP-CD63 mutants (B) or co-transfected with GFP-CD63ΔC12 and MT1-MMP mutants (D). The cell lysates were precipitated with anti-FLAG antibody (IP: FLAG), and analyzed by immunoblotting with anti-GFP antibody (Blot: GFP). The WCL were immunoblotted with anti–FLAG (Blot: FLAG) or anti-GFP (Blot: GFP) antibody. (C) a schematic representation of MT1-MMP mutants. SP, signal peptide; Pro, propeptide; Cat, catalytic domain; H, hinge region; Pex, hemopexin-like domain; TM, transmembrane domain; CP, cytoplasmic domain; FLAG, FLAG epitope. (E), HeLa cells were co-transfected with MT1F or MT1FΔPex and GFP or GFP-CD63 plasmids, and cultured in the presence of BB94 (1 μM) for 12 h. The cells were homogenized, and immunoblotted with anti-FLAG (Blot: FLAG) antibody.

FIG. 4. Role of cytoplasmic tails of MT1-MMP and CD63. **(**A) HeLa cells were co-transfected with MT1F or MT1FΔCP and either GFP, GFP-CD63 or GFP-CD63ΔC12 plasmids. The cell-surface MT1-MMP were labeled with anti-FLAG M2 antibody for 30 min at 4°C, and the

immunofluorescence staining was performed under non-permeabilized condition. Bar, 20 μm. (B) the values of fluorescence intensity per cell were determined by immunofluorescence staining. Data was collected from at least 30 cells from three independent experiments. Asterisk, $P < 0.0001$. Error bars indicate SD. (C) HeLa cells were co-transfected with MT1F or MT1FΔCP, MMP-2, and GFP or GFP-CD63 plasmids as described above. The conditioned media were analyzed by gelatin zymography.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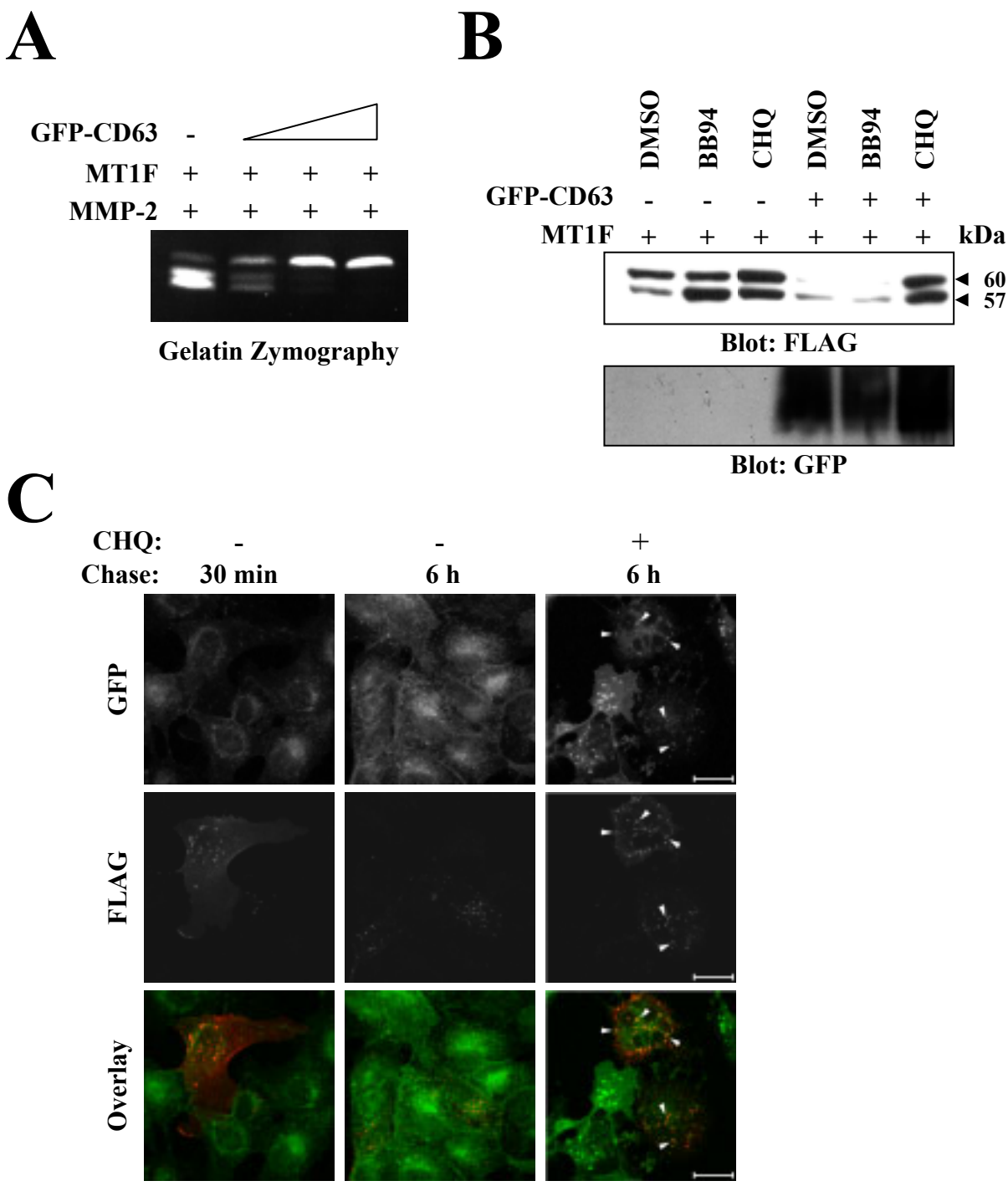
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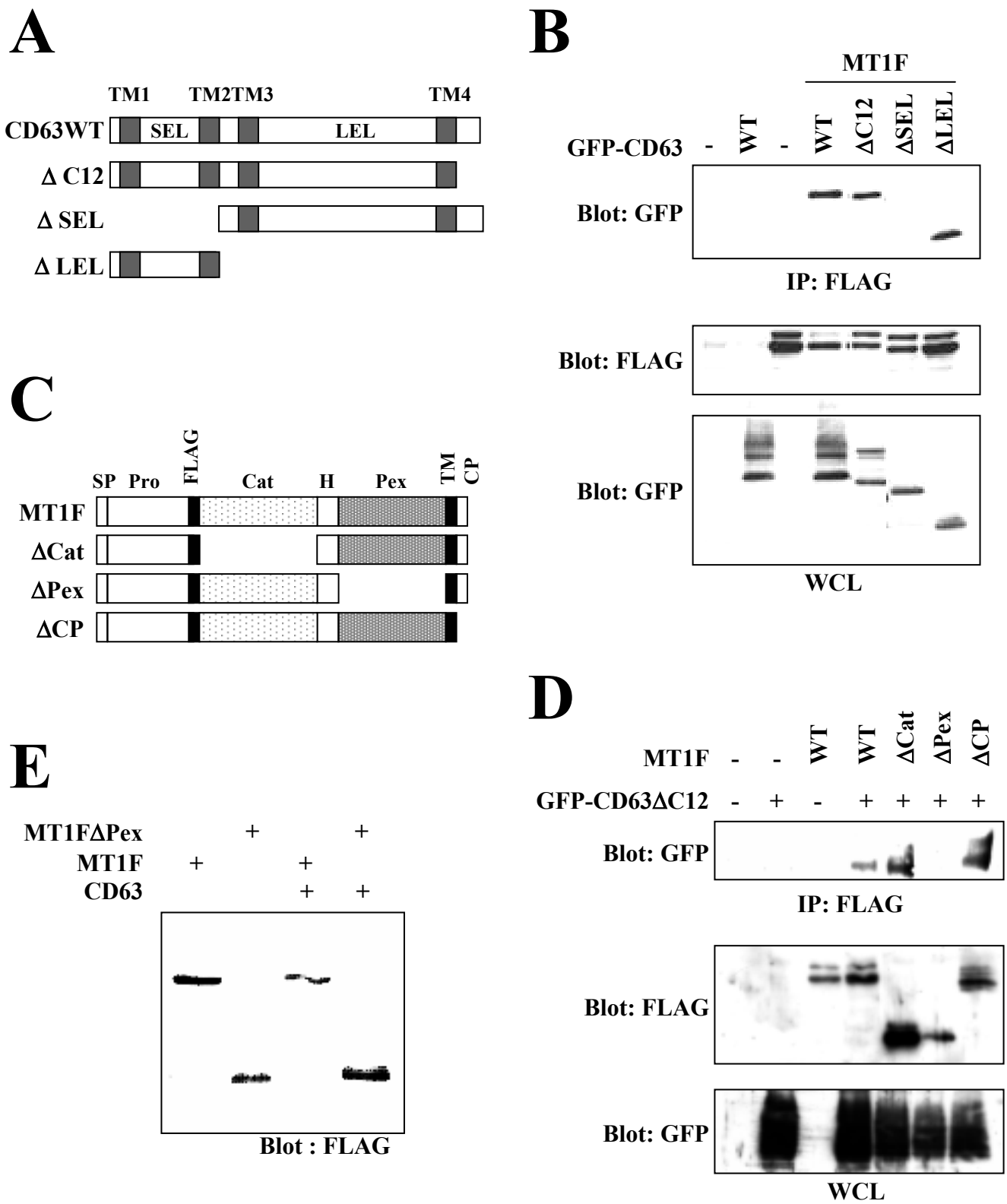
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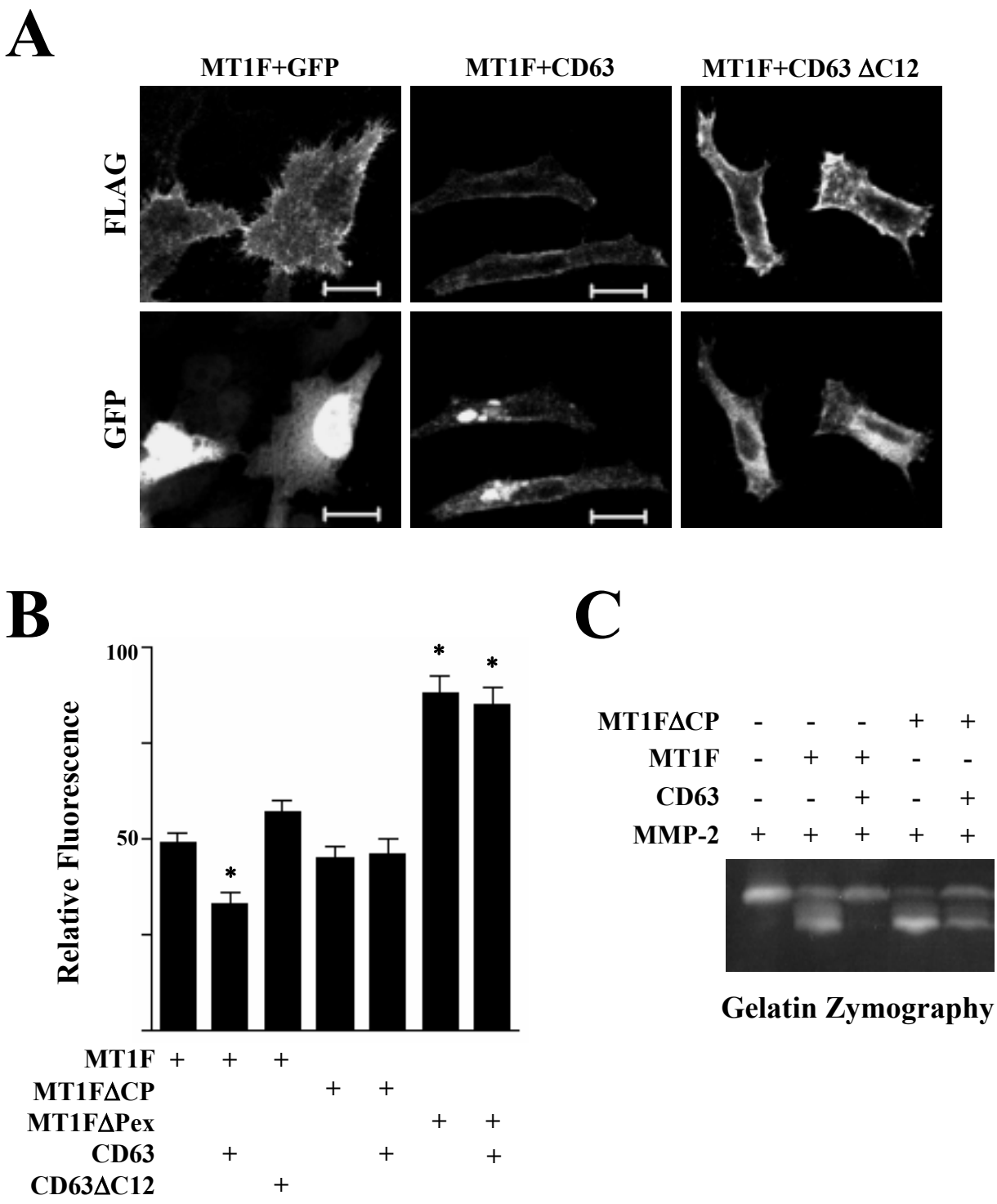
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