Proteasomal Degradation of the Nuclear Targeting Growth Factor Midkine*

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It is widely held that growth factor signaling is terminated by lysosomal degradation of its activated receptor and the endocytosed growth factor is transported to lysosomes. Nuclear targeting is another important pathway through which signals of growth factors are mediated. However, mechanisms underlying desensitization of nuclear targeting growth factors are poorly understood. Here we report that the nuclear targeting pathway is down-regulated by the proteasome system. Degradation of endocytosed midkine, a heparin-binding growth factor, was suppressed by both proteasome and lysosome inhibitors to similar extents. By contrast, a proteasome inhibitor, but not lysosome ones, accelerated the nuclear accumulation of midkine. An expression vector of signal sequence-less midkine, which is produced in the cytosol, was constructed because endocytosed midkine may be translocated to the cytosol from cellular compartments before entering the nucleus. The cytosol-produced midkine underwent proteasomal degradation and accumulation in the nucleus as did the endocytosed midkine. It was polyubiquitinated, and its nuclear accumulation was enhanced by a proteasome inhibitor. We further dissected the midkine molecule to investigate roles in degradation and trafficking. The N-terminal half-domain of midkine was significantly more susceptible to proteasomal degradation, whereas the C-terminal half-domain was sufficient for nuclear localization. Together, these data highlight the desensitization of nuclear targeting by growth factors and indicate a critical role of the proteasome system in it.

Because the proteasome system plays a critical role in endosome-to-lysosome trafficking (1–7), it may indirectly regulate the lysosomal degradation of receptors. In addition to the classical desensitization described above, an additional route, namely nuclear targeting by growth factors, should be considered. In this case, signaling is mediated directly by the growth factor and not via the cell surface receptor. Therefore, desensitization occurs only when the growth factor is degraded.

Mounting evidence indicates that nuclear targeting by growth factors plays an indispensable role in their biological activities. For example, the nuclear localization of fibroblast growth factor 1 and Schwannoma-derived growth factor is necessary for their mitogenic activity (8–10). Increases in ribosomal gene transcription and cell proliferation are tightly correlated to the nuclear translocation of fibroblast growth factor-2 (11, 12). Thus, signals from cell surface receptors and ligands translocated to the nucleus cooperate and play roles in the biological activities of many growth factors.

Midkine (MK)1 was first discovered as the product of a retinoic acid-responsive gene during the differentiation of embryonal carcinoma cells (13, 14). MK promotes neuronal survival (15–17), transforms NIH 3T3 cells (18), and is up-regulated during the development of human carcinomas and cerebral infarction (19–22). MK antisense oligonucleotide inhibits tumor growth in vivo (23). Furthermore, two animal models, arterial restenosis and interstitial nephritis, revealed that MK plays a critical role in the inflammation in these diseases, causing a more severe phenotype in wild-type mice as compared with that in MK-deficient mice (24, 25). Thus, MK is pivotal in neuronal survival, carcinoma development, and inflammation. Recently, we identified low density lipoprotein receptor-related protein (LRP) as the MK receptor, which is needed for MK-mediated neuronal cell survival (26). MK internalization is completely dependent on LRP, and internalized MK is translocated to the nucleus (27). This nuclear targeting is necessary for the full activity of MK in promoting cell survival (27).

It has long been accepted that endocytosed growth factors are transported to lysosomes where they are degraded. However, we demonstrate here that besides the lysosomal system, the proteasomal system is directly involved in the degradation of an endocytosed growth factor. Moreover, proteasome inhibitors specifically enhance nuclear accumulation of the growth

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1 The abbreviations used are: MK, midkine; LRP, low density lipoprotein receptor-related protein; HA, hemagglutinin; ER, endoplasmic reticulum; MEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; FL, full-length; SSFL, signal sequence-containing full-length; aa, amino acid; SSAC, signal sequence-containing C-terminal half-deletion mutant; GFP, green fluorescent protein; ER, endoplasmic reticulum.

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factor. This is the first demonstration that the proteasomal system is the key regulator of desensitization of signaling by nuclear targeting growth factors.

**Experimental Procedures**

**Reagents**—Yeast-produced human midkine protein was a generous gift from S. Sakuma (Cell Signals Inc., Yokohama, Japan). [125I]midkine iodide and dimethyl sulfoxide were from Sigma. The anti-heparin cofactor II monoclonal antibody (3F10) was purchased from Proteinnetworks. The fluorescein isothiocyanate-conjugated anti-rat IgG antibody, protease inhibitors, and chloramine-T were from Bio-Rad. Antibacterial peptide T 2 (an inhibitor of NF-κB signaling by retaining NF-κB in the cytosol) and histone H2A (a histone protein of the nucleosome), anti-εEF2 (eukaryotic elongation factor 2, a cytosolic protein with polypeptidyl-tRNA translocase activity), and anti-ε-Jun (a nuclear protein, a component of the transcription factor AP-1) were from Cell Signaling. The Lab-Tek chamber slides were from Nunc.

**Inhibitors**—MG132 (the final concentration used being 20 μM, Calbiochem), chlortoacticyanate β-lactone (20 μM, Biochem), and bafilomycin A1 (200 nM, Sigma) were dissolved in dimethyl sulfoxide. Brefeldin A (10 μg/ml, Sigma) was dissolved in ethanol, and ammonium chloride (20 mM, Wako, Osaka, Japan) and chloroquine (100 μM, Sigma) were dissolved in PBS. An equal volume of each solvent was used as the control vehicle. Inhibitors were added for preincubation and maintained during the pulse phase of metabolic labeling or [125I]midkine binding and throughout the chase unless specified.

**Cell Culture**—Mouse embryonic fibroblasts, MEF1 (LRP+/−, simian virus 40-transfected, CRL-2214) (28), and COS7 were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

**Radioiodination of MK**—The purified midkine protein was radioiodinated by the chloramine-T method. 10 μg of midkine in 90 μl of 50 mM sodium phosphate buffer, pH 7.2, was added to 1 mCi of [125I]sodium and 50 μl of a freshly prepared solution of chloramine-T (1 mg/ml in 50 mM sodium phosphate buffer, pH 7.2, Wako). After 1 min at 20 °C, the reaction was stopped by adding 50 μl of 5 mM sodium bi sulfite and 100 μl of 150 μM potassium iodide. Free [125I]sodium was separated from [125I]midkine by chromatography on Sephadex G-25 (PD10, Amersham Biosciences) equilibrated with 50 mM sodium phosphate buffer, pH 7.2, containing 0.25% bovine serum albumin. An analysis of [125I]MK by SDS-PAGE followed by autoradiography revealed a single band of 17 kDa. The specific activity of [125I]MK was determined to be 5 × 107 cpm/μg.

**Kinetic Analysis of Endocytosis and Degradation and Cell Fractionation**—MEF1 cells were preincubated for 60 min at 37 °C in culture medium containing an inhibitor or vehicle. The binding of [125I]MK was carried out for 1 h at 4 °C with the addition of 20 ng of [125I]MK/ml to the medium. Unbound ligand was removed by washing with ice-cold culture medium, and midkine-bound cells were incubated for various times at 37 °C in prewarmed medium containing an inhibitor or vehicle. After incubation, the plates were quickly placed on ice and the medium was replaced with ice-cold PBS. To remove the ligand remaining on the cell surface, the cells were harvested by trypsinization (0.125% trypsin and 0.025% EDTA in PBS at 37 °C for 10 min) and then washed with ice-cold culture medium and subsequently pelleted by low speed centrifugation (800 × g for 5 min at 4 °C). After washing, the cell pellet was further washed three times with the same buffer to obtain nuclei. Microscopic analysis demonstrated the absence of cytoplasmic membranes and organelles in the nuclear fraction. The nuclei were solubilized by boiling in SDS sample buffer and then immediately cooled on ice and centrifuged at 14,000 × g for 10 min (nuclear fraction). Both fractions were analyzed by SDS-PAGE followed by autoradiography.

Cell fractionation into membrane, cytosol, and nuclear fractions was carried out as described previously (27). After harvesting by trypsinization, cells were washed with ice-cold culture medium and the nuclear preparation signals into Triton X-100 and then homogenized in the nuclear preparation buffer without Triton X-100. Microscopic analysis confirmed the absence of intact round cells. After removal of the nuclear pellet by low speed centrifugation (800 × g for 5 min at 4 °C), high speed centrifugation (100,000 × g for 1 h at 4 °C) was performed to separate membranes and vesicles from soluble proteins. The soluble fraction was collected and the nuclear fraction was collected. The pellet was further resuspended in the nuclear preparation buffer containing 0.5% Triton X-100 (membrane fraction). The nuclear pellet was further processed as described above to obtain the nuclear fraction.

**Plasmid Constructs and Transfection**—Human MK consists of 433 amino acids (32). Signal sequence-containing full-length (SSFL), signal sequence-containing C-terminal half-deletion mutant (SSAC, 1–81 aa) and signal sequence-containing C-terminal half-deletion mutant (SSAC, 1–81 aa) were designed. Furthermore, six signal sequence-less constructs were designed as follows: full-length (FL) (23–143 aa); ΔC (23–81 aa); ΔN (82–143 aa); ΔC2 (23–98 aa); ΔIop (23–107 aa); and ΔC3 (23–118 aa). All of the constructs were transfected with the HA epitope at the C-terminal end and then subcloned into the Hind III/XhoI sites of pCDNA3.1 neo (+). Signal sequence-less full-length and ΔC2 of MK were also subcloned into the Hind III/BamHI sites of pEFGP-N1 to generate GFP fusion protein expression vectors (FL-GFP and ΔC-GFP). Preparation of an expression plasmid encoding histidine-tagged ubiquitin was performed as described previously (33). Each vector was transiently transfected into cells with LipofectAMINE Plus following the manufacturer's protocol.

**Ubiquitination Assay**—Ubiquitination assaying of overexpressed MK was performed by the method described previously (33). COS7 cells were transfected with or without a histidine-tagged ubiquitin expression vector and a signal sequence-less MK FL expression vector. 36 h after transfection, the cells were treated with 20 μg MG132 for 8 h and then lysed as described above. 10 μg of each sample was subjected to SDS-PAGE followed with 100 mM EDTA and then analyzed by immunoblotting with anti-HA monoclonal antibody.

**Membrane Labeling, Immunoprecipitation, and Diagonal Electrophoresis**—COS7 cells were cotransfected with MK expression vectors and cultured for 36 h before experiments. Cells were washed with Met/Cys-free Dulbecco's modified Eagle's medium and then preincubated for 30 min in the same medium containing a proteasome or lysosome inhibitor or the vehicle. After three washes with Met/Cys-free Dulbecco's modified Eagle's medium, the cells were pulsed for 10 min by adding 150 μCi of [35S]Met/Cys in 1 ml of medium and then chased for the times indicated in the figures with complete Dulbecco's modified Eagle's medium containing a proteasome or lysosome inhibitor or the vehicle (34). At the end of a chase, the cells were quickly placed on ice, washed with ice-cold PBS, and then lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin/ml, and 2 μg of leupeptin/ml). The cell lysates were subjected to a 10-min centrifugation at 13,000 × g at 4 °C. Immunoprecipitation was performed for 4 h from 4°C to 1°C h by adding anti-HA monoclonal antibody and then protein G beads for an additional 2 h. The immunoprecipitate was extensively washed with lysis buffer and then resuspended in the sample buffer. Relevant bands obtained on SDS-PAGE followed by autoradiography were cut out and analyzed by BAS2000 (Fuji Film).

**Indirect Immunofluorescence Staining**—To visualize the subcellular localization of overexpressed midkine, indirect immunofluorescence staining was performed. For these experiments, MEF1 cells were grown on chamber slides and then transfected with MK expression vectors. One day after transfection, the cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 10 min at 4 °C, permeabilized with 4% Triton X-100 in PBS at 4 °C for 20 min, and then blocked with 1% bovine serum albumin in PBS at 4 °C for 30 min. The treated cells were stained first with anti-HA monoclonal antibody and then with the corresponding fluorochrome-conjugated secondary antibodies.
and then with fluorescein isothiocyanate-conjugated anti-rat IgG antibody. Finally, they were extensively washed with PBS and then examined under a confocal microscopic system (MRC1024 system, Bio-Rad). For counterstaining, detection of nuclei was performed with 0.5 μg of propidium iodide/ml, which detected double-stranded nucleic acids.

RESULTS

Both Proteasome and Lysosome Inhibitors Suppress Degradation of Endocytosed MK—Exogenously added MK is internalized via LRP (27). A part of the endocytosed MK is further translocated to the nucleus, whereas the other part may be transported to the lysosomes (27). To verify the mechanism underlying the degradation of endocytosed MK, we first examined the effects of proteasome and lysosome inhibitors. When 125I-labeled MK was added to mouse embryonic fibroblast MEF1 cells, which express endogenous LRP (28), internalized MK reached the maximal level within 15 min and then decreased in a time-dependent manner (Fig. 1, B and D, closed squares). Consistently, the trichloroacetic acid-soluble 125I counts in the medium, which represent the degradation products, increased (Fig. 1, A and C, closed squares). The proteasome inhibitor MG132 and the lysosome inhibitor ammonium chloride suppressed the degradation of endocytosed MK to similar extents (Fig. 1, open squares). Because the proteasome system is involved in endosome-to-lysosome trafficking (1–7), these data do not necessarily indicate that proteasomes directly regulate the degradation of endocytosed MK. Thus, we further investigated the effects of these inhibitors on the intracellular trafficking of endocytosed MK.

Proteasome Inhibitor MG132 Accelerates Nuclear Accumulation of MK—After almost complete elimination of cell surface-bound 125I-labeled MK by trypsinization as described previously (27), cells were fractionated into post-nuclear and nuclear fractions. Consistent with the results in Fig. 1, the post-nuclear fraction showed similar suppressive effects of proteasome inhibitor MG132 and lysosome inhibitors ammonium chloride, bafilomycin, and chloroquine upon MK degradation (Fig. 2 A). By contrast, only MG132 strongly suppressed MK degradation in the nuclear fraction (Fig. 2B). These data suggest that degradation of endocytosed MK destined for the nucleus is directly controlled by the proteasome system. The Golgi-endoplasmic...
reticulum transport inhibitor brefeldin did not have any effect on MK degradation (Fig. 2).

**Cytosol-produced MK Is Polyubiquitinated**—In a previous study (27), we demonstrated that the nucleocytoplasmic shuttle protein nucleolin binds to endocytosed midkine. This binding can only be seen in LRP⁺/⁺ cells, which can endocytose midkine, but not LRP⁻/⁻ cells that cannot endocytose it. Although nucleolin can be detected in both membrane and cytosol fractions, only cytosolic nucleolin binds to midkine. Furthermore, nuclear localization signal-less nucleolin is retained in the cytosol and traps midkine there, leading to inhibition of the nuclear localization and cytoprotective activity of midkine. These data strongly suggest that midkine enters the cytosol across the membranes of intracellular vesicles after endocytosis.

To further investigate intracellular trafficking of endocytosed MK, here we monitored the exogenously added ¹²⁵I-labeled midkine in each cellular fraction. We first subjected the cell fractions to Western blot analysis to verify an accuracy of the fractionation procedure employed. IxB and eEF2 were mainly detected in the cytosol fraction (Fig. 3A). Calnexin (a membrane protein of the endoplasmic reticulum (ER) with chaperone activity) was mainly detected in the membrane fraction (Fig. 3A). c-Jun and histone H2A were detected only in the nuclear fraction (Fig. 3A).

The amount of endocytosed ¹²⁵I-labeled midkine reached the peak at 5 min in the membrane fraction, 15 min in the cytosol fraction, and 30 min in the nuclear fraction (Fig. 3, B–D). Taken together, it is most probable that endocytosed midkine enters the cytosol across the membranes of intracellular vesicles and is then translocated to the nucleus.

If the proteasome system is directly involved in MK degradation, endocytosed MK must be polyubiquitinated. However, there has been no report showing polyubiquitination of proteins translocated to the cytosol after endocytosis and we could not detect this for endocytosed MK (data not shown). This is probably due to that the absolute amount of the polyubiquitinated fraction of endocytosed proteins is too small to be detected as compared with the whole polyubiquitinated fraction of intracellular proteins. An expression vector of signal sequence (Met¹-Ala²² in human MK)-less MK was thus constructed. The signal sequence-less MK has the same amino acid composition and sequence as exogenous MK but cannot be secreted. Instead, it should be produced in the cytosol. Polyubiquitination of the signal sequence-less MK was detected and was enhanced in the presence of MG132 (Fig. 4).

**Signal Sequence-less MK Accumulates in the Nucleus**—Signal sequence-less MK was localized in the nucleus as reported for exogenously added and endocytosed MK (Fig. 5, A, FL), whereas signal sequence-containing MK was detected in the cytoplasm, probably in the ER where it is produced and eventually secreted (Fig. 5A, SSFL). Because signal sequence-less MK fused to green fluorescence protein also accumulated in the nucleus (Fig. 5B, FL-GFP), the MK moiety is a strong determinant of nuclear localization. The nuclear localization was also enhanced in the presence of MG132 (Fig. 5B, FL MG132), resembling the data in Fig. 2. Together, these results indicate that signal sequence-less MK represents the nucleus-destined fraction of internalized MK.

The MK molecule essentially consists of two domains, the N- and C-terminal half-domains (35). The N-terminal half-domain corresponds to Lys⁸²-Gly⁹¹, and the C-terminal half-domain corresponds to Ala¹⁰²-Asp¹⁴³ of human MK (32). To determine the domain responsible for nuclear localization, we constructed expression vectors corresponding to these domains. The N-terminal half-domain was barely detectable in the steady state (Fig. 6A, ΔC), whereas the C-terminal half-domain was fairly detectable (Fig. 6A, ΔN) and located in the nucleus (Fig. 5A, ΔN). The green fluorescence protein-fused ΔN also accumulated in the nucleus (Fig. 5B, ΔN-GFP). Therefore, the data suggest that the C-terminal half-domain is sufficient for the nuclear localization of internalized MK.

**The N-terminal Half of MK Is Significantly More Susceptible to Proteasomal Degradation**—The scarcely detectable expression of the N-terminal half-domain in the steady state (Fig. 6A, ΔC) prompted us to further investigate this phenomenon. According to the ternary structure of MK revealed by NMR (36), both the N- and C-terminal half-domains consist of three anti-parallel β-strands, respectively (Fig. 6C). The N-terminal half:
Because SSFL and SSΔC contain the signal sequence, they are translated into the ER and then secreted. In other words, they are protected from proteasomal attack by being compartmentalized in the ER and Golgi apparatus. In this regard, it is interesting that signal sequence-less FL and ΔC showed an apparent difference in half-life upon comparison with their signal sequence-containing counterparts, i.e. ΔC showed a much shorter half-life than SSΔC, whereas FL was comparable with SSFL and SSΔC (Fig. 6C). Thus, it is likely that the N-terminal half-domain is important for degradation when MK is in the cytosol or nucleus. As shown in Fig. 7, the proteasome but not lysosome inhibitors suppressed signal sequence-less MK constructs. Together, these results indicate that degradation of intracellular MK is regulated by the proteasome system and suggest that the N-terminal half-domain is the most important for this degradation, although the full molecule is necessary to obtain the maximal half-life.

**DISCUSSION**

The present results suggest that the termination of signaling by nuclear targeting growth factors is coordinated by fine mechanisms including the proteasome system. We demonstrated that MK is prone to proteasomal degradation. Because “off” of signaling is essential for life, the finding that nuclear targeting growth factors are prone to degradation seems reasonable. By contrast, toxins, such as cholera toxin and ricin, are resistant to proteasomal degradation, which is ascribed to a paucity of lysines. Lysine is the potential residue of ubiquitination (38, 39). MK contains many lysine residues (9 lysines (15%) among 59 residues in the N-terminal half; 14 lysines (23%) among 62 in the C-terminal half; 23 lysines (19%) among 121 in total). In this context, it is of note that cytosolic MK is associated with nucleolin or laminin-binding protein precursor (27, 40). To exert their functions effectively, nuclear targeting growth factors may associate with some partner proteins not only for proper transport to their destination (the nucleus) but also for escape from degradation machineries.

The C- but not the N-terminal half-domain of MK is evolutionally conserved from Drosophila to man, has the major heparin-binding sites, and is the major biologically functional domain (35). These results further differentiate the biological roles of these domains in terms of intracellular trafficking. For instance, the N-terminal half-domain is important for desensitization, whereas the C-terminal half-domain is sufficient for translocation to the nucleus. Because the full-length is necessary for the full stability of MK (Fig. 6), these domains may collaborate in MK trafficking and degradation with the aid of intracellular MK-associated protein.

It is important to determine the intracellular site of MK degradation in terms of the action mechanism of MK targeting to the nucleus. There is an increasing body of evidence that the proteasome system exists in both the cytosol and nucleus (41–43). Taking into consideration that MK is associated with nucleolin or laminin-binding protein precursor until it reaches the nucleus, it is possible that MK is attacked by proteasomes when MK completes its biological role in the nucleus. Supporting this possibility, a model of cyclic proteasome-mediated turnover of estrogen receptors on responsive promoters in the nucleus was recently established (44).

LRP belongs to the low density lipoprotein receptor family. Classically, proposed functions of these receptors are the endocytosis and delivery of a diverse array of ligands to lysosomes for degradation or catabolism (45). LRP exhibits the strongest endocytosis activity among the low density lipoprotein receptor family members (46). According to the recently revealed ternary structure of low density lipoprotein receptor, the ligand binding domain folds back at low pH so that its central modules

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**FIG. 4.** Polyubiquitination of intracellular MK. Polyubiquitination of signal sequence-less MK was examined as described under “Experimental Procedures.” Ub, ubiquitin; AP, affinity precipitation; Ni, nickel.

**FIG. 5.** Nuclear accumulation of signal sequence-less full and C-terminal half-MK. SSFL MK, MK FL, MK ΔN, MK FL-GFP, MK ΔN-GFP, or GFP was overexpressed, and then its intracellular localization was investigated. Signal sequence-less full MK was also used to determine the effect of MG132 on nuclear localization. Propidium iodide (PI) was used to stain nuclei. DMSO, Me2SO. Bar, 10 μm.

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| Lysate | AP-Ni beads |
|--------|-------------|
| His-Ub | –           |
| MK-HA(FL) | –   |
| MG132 | –           |

| Ub-MK conjugates | free MK |
|-----------------|---------|
| SSFL           | 25      |
| FL             | 37      |
| ΔN             | 30      |

**FIG. 6A** shows the steady state expression of each construct. SSFL and FL MK showed comparable expression. Compared with them, ΔN, ΔC2, Δloop, and ΔC3 showed weaker expression, whereas ΔC was scarcely visible. The half-lives were consistent with steady state expression (Fig. 6, B and C). It is of note that ΔC was fairly well translated as revealed upon pulse-chase labeling but was degraded very rapidly (Fig. 6, B and C), although steady state expression was barely detectable. Thus, it is unlikely that the low expression of the steady state of ΔC was due to mRNA instability caused by such as nonsense-mediated decay (37).
come into contact with the β-propeller domain, leading to the dissociation of ligands (47). Thus, it seems that LRP and its ligands may also become dissociated at low pH in endosomes. This idea is supported by the fact that LRP is frequently recycled back to the cell surface, whereas Pseudomonas exotoxin A, an LRP ligand, travels from endosomes to the trans-Golgi network and eventually to the ER where it is translocated to the cytosol (28). However, intracellular trafficking could depend on endocytosed ligands themselves and not receptors. Indeed, we found in the previous study (27) that exogenous MK and receptor-associated protein, an artificial LRP ligand, showed distinct trafficking (MK to the nucleus and receptor-associated protein to lysosomes). Fibroblast growth factor-1 enters the cytosol from vesicles possessing vacuolar proton pump (48). Continued binding of epidermal growth factor receptor in endosomes leads to sustained phosphorylation of the receptor and promotes proteasome-dependent trafficking of the receptor to lysosomes, whereas transforming growth factor-α dissociates from epidermal growth factor receptor in endosomes (7). It is not probable that internalized MK is released from the endoplasmic reticulum, because the Golgi-endoplasmic reticulum transport inhibitor brefeldin did not have any effect on MK degradation (Fig. 2). Thus, the exit vesicle (early endosomes, the ER, or others) for MK remains to be determined and the underlying molecular mechanism would be an interesting subject for further investigation.

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FIG. 6. Half-lives of MK. The steady state expression (A) and half-life (B and C) of each MK construct were monitored by means of Western blot analysis (A) and the pulse-chase metabolic labeling method (B and C).

FIG. 7. Proteasome-dependent degradation of signal sequence-less MK. The relative amount of each MK construct that remained in the cell was monitored by means of pulse-chase metabolic labeling after incubation of the indicated time in the absence (cont) or presence of lysosome inhibitor ammonium chloride (AC), lysosome inhibitor chloroquine (Cq), proteasome inhibitor clasto-lactocystin β-lactone (Lac), or proteasome inhibitor MG132 (MG). The value of time 0 was set at 100%.
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