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Processing of a fusion protein by endoprotease in COS-1 cells for secretion of mature peptide by using a chimeric expression vector

(furin/calcitonin precursor/Fc fragment)

YUN-CAI LIU*†, MAYUMI KAWAGISHI*, TOSHIFUMI MIKAYAMA*, YOSHIMASA INAGAKI*, TOSHIYUKI TAKEUCHI‡, AND HIDEYA OHASHI*

*Pharmaceutical Research Laboratory, Kirin Brewery Co. Ltd., and ‡Institute for Endocrinology, Gunma University, Maebashi 371, Japan; and †La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037

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ABSTRACT **The subtilisin-related proprotein convertase furin is expressed in various mammalian tissues. Expecting that COS-1 cells have a furin-like endoprotease, we constructed a fusion expression vector for production of a recombinant foreign protein having no signal peptide or a protein in truncated form into secreted mature protein. A cDNA fragment encoding N-terminal procalcitonin (pro-CT) of human calcitonin precursor was inserted into the mammalian expression vector pME18S. We used PCR techniques to generate four kinds of cDNAs encoding the C terminus of the pro-CT with Arg residues at P4 (Arg-Xaa-Lys-Arg), P6 (Arg-Xaa-Xaa-Xaa-Lys-Arg), or both (Arg-Xaa-Arg-Xaa-Lys-Arg), in addition to the Lys-Arg motif at the cleavage site, in order to determine the conditions for efficient processing in nonendocrine cells, such as COS-1 cells. The cDNA coding for the Fc fragment of human immunoglobulin G1 was fused in-frame to the cDNA encoding pro-CT at its C terminus. Upon transfection of the chimeric plasmids into COS-1 cells, almost all of the fusion protein with the Arg residues at both P4 and P6 were processed into secreted Fc product, even without cotransfection of furin. These results indicate that COS-1 cells have a furin-like endoprotease and suggest that pro-CT, with the Arg residues at both P4 and P6, can be used as a carrier peptide for expression of a foreign protein having no signal peptide or a protein in truncated form in COS-1 cells.**

Many secretory proteins including polypeptide hormones, growth factors, and plasma proteins are synthesized as precursor proteins and undergo posttranslational proteolytic processing, which is frequently required for their biological activities. Prohormones of neuroendocrine origin often have a paired dibasic motif (Lys/Arg-Arg) at the cleavage sites within the precursors, whereas other secretory proteins have the Arg-Xaa-Lys/Arg-Arg motif, which contains an additional Arg residue at the P4 position (1, 2). The differences in the cleavage recognition sequences imply that different processing enzymes should exist for proteolytic specificity. It was not until recently that several processing enzymes have been identified and characterized from mammalian sources. One member of the processing enzymes, furin, was originally identified as a homologue of the yeast propeptide processing enzyme Kex2 (3, 4) and represents a counterpart of the prohormone convertases (PCs) of neuroendocrine origin (5, 6). In contrast to PC2 and PC3, which are expressed specifically in pancreatic islet, pituitary, and other neuroendocrine cells (7, 8), furin is expressed in many tissues and nonneuroendocrine cells (9–11). This enzyme appears to be predominantly localized to the Golgi region (10, 12), suggesting that furin or a furin-like enzyme, such as PACE4 (13, 14), is involved in the cleavage of proproteins secreted via the constitutive pathway (1).

Progress in biotechnology has made it possible to produce foreign proteins in cultured mammalian cells by expressing their genes or cDNAs under the direction of an appropriate promoter. However, proteins lacking a signal peptide may remain in the intracellular space according to their subcellular destinations, and proteins in a truncated form cannot be expressed unless they carry a correct translation initiation sequence, ATG. In the present study, attempts were made to construct a mammalian version of fusion protein vector with which a recombinant protein lacking signal peptide or that of a truncated form can be produced and secreted from transfected cells. Our approach was to fuse cDNA for a prepro-region of a protein precursor with the cDNA encoding the truncated peptide for expression of a fusion protein in COS-1 cells and to use furin or furin-like protease for intracellular cleavage of the fusion protein and subsequent secretion of the mature peptide. Since the N-terminal proregion of human calcitonin precursor (pro-CT) has Arg-Ser-Lys-Arg at its C terminus (15), we used this proregion as a carrier peptide for foreign proteins. Four different cDNA mutants were made in order to modify the amino acid sequences of the pro-CT C terminus into different cleavage motifs that can be recognized by furin. After the furin cleavage site, a multiple cloning site was created for insertion of a desired cDNA, followed by a polyadenylylation site of simian virus 40 (SV40) DNA. This pro-CT cDNA construct was directed under the control of a strong chimeric promoter of SV40 and retrovirus, SRα (16). In the present experiment, cDNA encoding the Fc fragment of human IgG1 was inserted in-frame into this vector, and this recombinant chimeric cDNA construct was introduced into COS-1 cells. The results show that the fusion protein with Arg residues at both P4 and P6 (Arg-Xaa-Arg-Xaa-Lys-Arg) at the cleavage site is processed most efficiently for secretion of Fc fragment, even in the absence of furin cotransfection, suggesting the usefulness of pro-CT with the P4 and P6 Arg residues as a carrier peptide in the fusion expression vector.

MATERIALS AND METHODS

Construction of the Fusion Expression Vector. The cDNA fragment encoding the signal peptide and pro-CT of a human calcitonin precursor was amplified by PCR with a human calcitonin cDNA used as the template (Y.-C.L., unpublished data). One 5′ end primer (CT1) and four different 3′ end primers (CT2, CT3, CT4, and CT5) were used for PCR, as listed in Fig. 1. The primers CT2, CT3, CT4, and CT5 were

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Abbreviations: pro-CT, N-terminal proregion of human calcitonin precursor; Pc, prohormone convertase; SV40, simian virus 40.

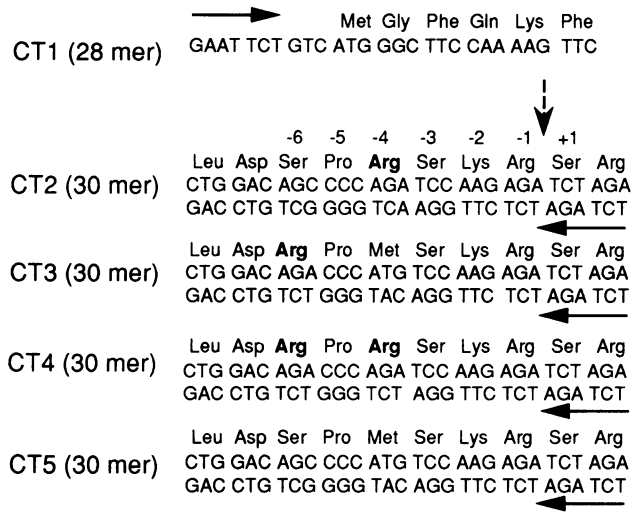

FIG. 1. Oligonucleotide primers for PCR amplification of human pro-CT. Arrows with solid lines indicate the synthesized primer in the 5′ to 3′ direction. CT1 encodes the N terminus of pro-CT and the four other primers encode the C terminus of pro-CT. Arrow with dashed line indicates the cleavage site by endoprotease. The nucleotide sequence AGATCTAGA in CT2–CT5 is recognized by *Bgl* II and *Xba* I.

made to carry several basic residues in different arrangements in order to examine the processing efficiency of putative endoprotease in COS-1 cells. An *Eco*RI restriction site was introduced into CT1, and *Bgl* II plus *Xba* I restriction sites were introduced into the four other primers. After generating the four corresponding pro-CT cDNA fragments, they were subcloned into the *Eco*RI and *Xba* I sites of the plasmid pBluescript II KS(+) (Stratagene). Since *Not* I became a neighbor restriction site after *Xba* I on this plasmid vector, the pro-CT cDNAs were excised with *Eco*RI and *Not* I. Then, each of the four different pro-CT cDNAs with mutated 3′ ends was inserted into the mammalian expression vector pME18S, which carries a strong chimeric promoter of SV40 and retrovirus, SRα (16), to generate a plasmid vector, pMEpro-CT (Fig. 2).

Cloning of cDNAs. The Fc cDNA of human IgG1 was obtained by PCR with two 28-nucleotide primers, G1 and G2, and with poly(A)$^+$ mRNA from human leukemia cell line

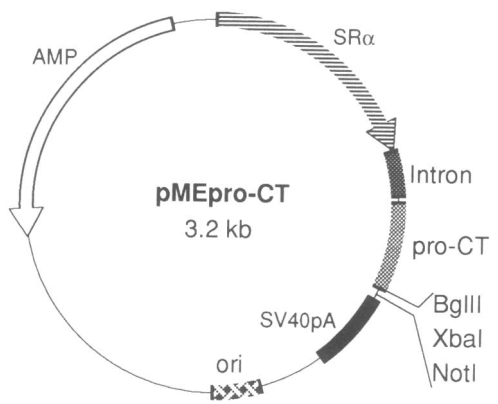

FIG. 2. Structure of the fusion expression vector pMEpro-CT. Plasmid pME18S is the backbone for construction of the vector. An *Eco*RI/*Not* I fragment encoding the signal peptide and the N-terminal pro-CT was inserted into the *Eco*RI and *Not* I sites of pME18S. The proteolytic cleavage motif was included in the C terminus of pro-CT, followed by the multicloning sites *Bgl* II/*Xba* I/*Not* I for insertion of foreign genes of interest into pro-CT. SRα, SV40-derived promoter; intron, region for mRNA splicing; SV40pA, polyadenylylation signal from SV40.

ARH-77 (American Type Culture Collection, CRL1621) as a template. The 5′ end primer G1 (5′-CAGATCTCACACATGCCCACCGTGCCCA-3′) and the 3′ end primer G2 (5′-GGCGGCCGCCGCACTCATTTACCCGGAG-3′) contained a *Bgl* II site and a *Not* I site (underlined), respectively. The Fc cDNA thus obtained was subcloned into the TA cloning vector pCR100 (Invitrogen). The cDNA fragment was sequenced by using an Applied Biosystems model 370A sequencer. The Fc cDNA encoded a sequence of His-Thr-Cys-Pro-Pro-Cys-Pro-Ala- at the N terminus, flanked by Ser as a linker, and encoded two Cys residues in the hinge region (17). The cDNA was inserted in-frame after the pro-CT into the pMEpro-CT.

Human furin cDNA was cloned by the same PCR amplification method. Poly(A)$^+$ mRNA was prepared from human bladder carcinoma cell line HT1376 (American Type Culture Collection, CRL1472) and was reverse-transcribed into cDNA, which was used as a PCR template. Six oligonucleotide primers (Fig. 3) were prepared based on the published furin cDNA sequence (3, 4) by an Applied Biosystems model 394 DNA synthesizer. Three cDNA fragments, covering the coding sequences of 1–951, 922–1604, and 1565–2385 bp of human furin DNA, respectively, were purified from the corresponding PCR products. The cDNA fragment encoding the N-terminal protein sequence was annealed with the adjoining cDNA fragment by making use of a 27-bp overlap between the two fragments. The resulting cDNA mixture was reamplified by using primers corresponding to the 5′ end of the first fragment and the 3′ end of the second fragment. The 1.6-kb cDNA thus derived was ligated via the *Bsp*HI site with the third cDNA fragment encoding the remaining C-terminal furin. The whole cDNA construct was subcloned into the TA cloning vector pCR1000 (Invitrogen). Human furin cDNA sequences were determined by using the Sequenase kit (United States Biochemical). Restriction site mapping and partial sequence analysis revealed that the cloned cDNA was identical to the human furin previously reported (3, 4). An *Eco*RI/*Not* I fragment containing the full-length human furin was cloned into the mammalian expression vector pEFneo, which was generated by inserting a neo-expression unit into a modified pEF-BOS (18).

The primers described above for human furin were also used for detection of endogenous furin expression in COS-1 cells. To identify the mRNA expression of PACE4 in COS-1 cells, PCR was performed by using a sense primer of 27-mers (5′-ATTTACAGTGCCAGCTGGGGGCCGGAC-3′) and an antisense primer of 27-mers (5′-CAGGTGCTGGACGTCCCTCCAGGTTAA-3′) corresponding to human PACE4 (13).

Transfection and Expression Analysis. COS-1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 5% fetal calf serum in a humidified incubator at 37°C under 5% CO_2/95% air. Upon transfection, the cells were washed twice with serum-free IMDM and were further cultured in IMDM containing an additive mixture of bovine insulin (5 μg/ml), human transferrin (5 μg/ml), monoethanolamine (10 μM), and sodium selenite (25 μM). Plasmid DNA was transfected into COS-1 cells by using the Trans-

F1 (25 mer) 5′-CCCCATGGAGCTGAGGCCCTGGTTG-3′

F2 (28 mer) 5′-GATGGACAGCGTGTAGATACTGTTGGTG-3′

F3 (28 mer) 5′-ACACCAACAGTATCTACACGCTGTCCAT-3′

F4 (28 mer) 5′-GTCATGAAGGCCCAGTCATTAAACCCAT-3′

F5 (21mer) 5′-ACTACTCCGCAGATGGGTTTA-3′

F6 (21 mer) 5′-TCAGAGGGCGCTCTGGTCTTT-3′

FIG. 3. Oligonucleotide primers used for PCR cloning of human furin.

fectam kit according to the protocol provided by the manufacturer (IBF Technics, Columbia, MD). After incubation for 3 more days, the culture medium was collected and concentrated 2- to 5-fold with a Centricon-3 microconcentrator (Amicon). Samples were resolved by 4–20% gradient SDS/PAGE and were electrotransferred onto a Hybond nitrocellulose membrane (Amersham). For Western blotting, rabbit anti-human IgG (γ specific) antibodies conjugated with alkaline phosphatase (Dako) were reacted with membrane-bound proteins, followed by addition of the enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and *p*-nitroblue tetrazolium chloride for color development.

N-Terminal Peptide Sequence Analysis. Supernatant from transfected COS-1 cells was concentrated and the Fc fragment in the supernatant was purified by using a protein A-Sepharose column (Pierce). The purified Fc fragment was separated by SDS/PAGE, electrotransferred onto a poly(vinylidene difluoride) membrane, and stained with Ponceau S. The band corresponding to the processed form of Fc protein was subjected to automated Edman degradation in a PSQ-1 gas-phase sequencer (Shimadzu).

RESULTS

Vector Construction. The mammalian fusion expression vector pMEpro-CT was constructed by utilizing the signal peptide and the N-terminal region of human pro-CT as a carrier peptide for foreign proteins. As shown in Fig. 2, this vector basically consists of (*i*) a chimeric promoter, SRα, fused by SV40 promoter plus the R region of the long terminal repeat of the retrovirus (16); (*ii*) pro-CT; and (*iii*) SV40 early poly(A) addition signal. A multiple cloning site for *Bgl* II, *Xba* I, and *Not* I was attached, following the C-terminal proteolytic cleavage site of pro-CT, for insertion of a foreign gene of interest. The nucleotide sequences (AGATCTAGA) recognized by *Bgl* II and *Xba* I encode Arg-Ser-Arg residues in the reading frame (cf. Fig. 1). Therefore, foreign cDNA can be introduced and fused with the pro-CT cDNA according to this frame.

Secretion and Cleavage of the Chimeric Proteins. To test whether the fusion protein comprising the pro-CT region and the Fc fragment could be produced in a secreted form from the plasmid-transfected COS-1 cells, we used the chimeric plasmid containing cDNA encoding pro-CT with only dibasic Lys-Arg residues. Immunoblot analysis of the culture supernatant of the transfected cells with anti-human IgG showed that the fusion protein was detected predominantly at a molecular size of ≈66 kDa under nonreducing conditions, with a minor band of ≈33 kDa (Fig. 4). Under reducing conditions, only one protein band could be detected at a molecular size of ≈37 kDa. Specific immunostaining for the Fc fragment was not observed in the culture supernatant of the cells transfected with plasmid DNA of pMEpro-CT alone. Based on the findings that the molecular size of the major protein in the nonreduced form was approximately twice that found under reducing conditions, we hypothesized that the fusion protein must be synthesized as a dimer protein. Since no Cys residue exists in the pro-CT region, the dimer could arise from the disulfide bonds formed between the Cys residues in the hinge region of the Fc fragment.

We proceeded to determine the processing efficiency by transfecting the chimeric cDNAs with different cleavage motifs into COS-1 cells. Immunoblot analysis showed that the 30-kDa Fc fragment, released by proper proteolytic cleavage, was detected in the medium expressing the pro-CT with Arg residue(s) at P4 and at both P4 and P6, but it was barely detected where there was a residue at P6 alone and not at P4 (Fig. 5). Among these three constructs, the pro-CT with the Arg residues at both P4 and P6 was processed most efficiently to the mature Fc protein. As expected, no cleavage

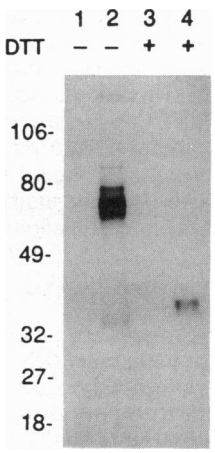

FIG. 4. Secretion of the fusion protein into culture supernatant of COS-1 cells transfected with plasmid cDNAs. Samples were resolved by SDS/PAGE under nonreducing [DTT (dithiothreitol) −] or reducing (DTT +) conditions and were then electrotransferred onto a nitrocellulose membrane, followed by immunoreaction with anti-human IgG antiserum conjugated with alkaline phosphatase. Lanes: 1 and 3, culture supernatant of COS-1 cells transfected with the nonrecombinant plasmid pME18S; 2 and 4, culture supernatant of COS-1 cells transfected with the plasmid cDNA encoding the Fc fragment and pro-CT containing the Lys-Arg motif.

was detected when a fusion protein was expressed with the Lys-Arg motif at P1 and P2 alone.

The Fc fragment obtained by transfection of the construct with the Arg residues at both P4 and P6 was purified. As expected, essentially all Fc fragment detected in the COS-1 cells bound to protein A-Sepharose and was recovered by elution with 0.1 M glycine hydrochloride (pH 2.8). To confirm the cleavage site at the fusion protein, the purified Fc fragment was analyzed by SDS/PAGE under reducing conditions, transferred to poly(vinylidene difluoride) membrane, and subjected to N-terminal sequence analysis. The first 10 N-terminal amino acids were Ser-His-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro. This sequence was consistent with the sequence encoded by the Fc cDNA in the fusion plasmid, indicating that the fusion protein was cleaved, as predicted, at the junction of the C terminus, Arg, of pro-CT and Ser, a linker for the Fc fragment.

To determine whether cotransfection with furin could improve processing efficiency, a human furin cDNA was isolated from the cDNA library made of a human bladder cell

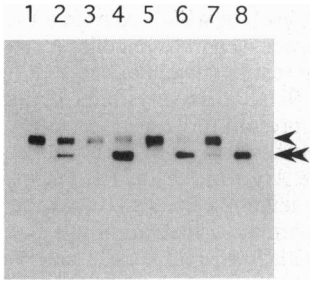

FIG. 5. Immunoblot analysis of culture supernatant from COS-1 cells transfected with four kinds of chimeric plasmids in the absence (lanes 1–4) or presence (lanes 5–8) of furin cotransfection. Samples under reducing conditions were processed as described in Fig. 4. Lanes: 1 and 5, plasmid cDNA encoding Lys-Arg motif at the cleavage site; 2 and 6, fusion protein with Arg at P4 in addition to the Lys-Arg motif; 3 and 7, fusion protein with Arg at P6 in addition to the Lys-Arg motif; 4 and 8, fusion protein with two Arg residues at both P4 and P6 in addition to the Lys-Arg motif. Single arrowhead, chimeric protein of pro-CT and Fc fragment; double arrowhead, processed Fc fragment.

line HT1376. Predictably, cotransfection with pEFneo-furin increased the cleavage efficiency, as shown in Fig. 5. Cotransfection of the endoprotease furin enhanced cleavage of the fusion protein with additional Arg at P4 or P6. Notably, the fusion protein with additional Arg residues at both P4 and P6 was completely processed into mature Fc without any trace of the fusion protein. It should be noted that fusion protein with the dibasic Lys-Arg motif alone remained unprocessed, even with furin cotransfection.

DISCUSSION

Eukaryotic secretory proteins are generally extended at their N terminus by signal sequences, which cross the endoplasmic reticulum and then are cleaved off the polypeptides. Secretory proteins are then transported to the Golgi apparatus, reach secretory vesicles, and finally are released into the extracellular space (19). In contrast to the secretory proteins, intracellular proteins are synthesized without signal sequences and are generally not secreted. For purification and characterization of a foreign protein lacking signal sequence or a protein in truncated form, it will be useful to express the protein in secreted form in cultured mammalian cells. In the present study, we attempted to construct a fusion vector by using the N-terminal proregion of human calcitonin as a carrier peptide for a foreign protein.

Calcitonin is synthesized as a large precursor in endocrine C cells of the thyroid. The calcitonin precursor consists of the N-terminal proregion (pro-CT), calcitonin, and the C-terminal extension peptide, which undergoes proteolytic cleavage at the flanking dibasic sites and an amidation reaction at the C-terminal glycine to become a biologically active peptide (15, 20). It has been believed that procalcitonin is processed by PC(s), which recognize the Lys-Arg cleavage site, because PCs are present in neuroendocrine tissues and have been identified for the processing of prohormones such as proopiomelanocortin (21), prorenin (22), and proinsulin (23). However, the amino acid sequence of the C terminus of human pro-CT is Arg-Ser-Lys-Arg, which should be recognized by the nonendocrine cell endoprotease furin. Thus, we reasoned that human pro-CT may be used as a carrier peptide for a foreign protein, so that fusion protein will be cleaved intracellularly at the junctional site, and foreign protein will be released into culture medium. Indeed, transfection of COS-1 cells with pro-CT cDNA fused with Fc cDNA resulted in secretion of Fc fragment; however, processing of the fusion protein in COS-1 cells was inefficient (Fig. 5, lane 2). The results are in agreement with previous reports that only a small fraction of pro-von Willebrand factor (24) and rat proinsulin I (23), both of which contain an Arg-Xaa-Lys-Arg sequence, was processed in COS cells. As expected, cotransfection with furin resulted in enhanced secretion of processed mature protein in all three systems including pro-CT–Fc fragment fusion protein (cf. Fig. 5, lane 6).

In view of these findings, the C-terminal end of pro-CT was designed to have Arg residues at P4, P6, or both P4 and P6. The reasons for selecting P6 are (*i*) that the cleavage site of proalbumin has Arg at P6 instead of at P4 but cotransfection of proalbumin with furin into COS-1 cells resulted in secretion of processed albumin (12), and (*ii*) that proparathyroid hormone has a basic residue at P6 and is processed into the mature secretory form of the peptide in *Xenopus* oocytes (25), which generally do not process a simple pair of basic residues such as Lys-Arg (26). Furthermore, sequence alignment shows that the C-terminal ends of chicken and salmon pro-CTs have an Arg residue at P6. In the present study, however, COS-1 cells revealed little, if any, processing of the pro-CT fusion protein with Arg at P6 (Fig. 5, lane 3). It was also found that cotransfection with furin converted only a fraction of the fusion protein into mature Fc fragments.

Of particular interest in the present study is the finding that the fusion protein with Arg residues at both P4 and P6 was processed almost completely even in the absence of furin cotransfection, resulting in the predominant production of mature Fc fragment (Fig. 5, lane 4). The mature Fc fragment secreted from transfected COS-1 cells bound to both protein A-Sepharose and anti-human IgG, indicating that the fragment has a proper functional conformation. Furthermore, N-terminal peptide sequencing showed that the fusion protein was precisely cleaved at the junction of pro-CT and the Fc fragment. This work demonstrates that COS-1 cells efficiently process the cleavage sequence Arg-Xaa-Arg-Xaa-Lys-Arg. It remains to be determined whether the fusion protein with P4 and P6 Arg residues is processed by the same enzyme as that which recognized the cleavage site with Arg at P4 alone or by a different endogenous protease, as discussed by Steiner *et al.* (1). It is possible that the enhanced efficiency of processing for the cleavage site with both P4 and P6 Arg may be the result of conformational changes that facilitate recognition and processing of the cleavage site by the enzyme in COS-1 cells. Rehemtulla *et al.* (27) reported that neither culture supernatant nor cell lysate of COS-1 cells contained a detectable amount of furin, as determined by Western blot using rabbit anti-human furin antibodies. Attempts were therefore made to detect furin mRNA in COS-1 cells by the PCR method using six human furin primers listed in Fig. 3; however, no furin mRNA was detected (results not shown). The results suggest that the processing endopeptidase in COS-1 cells is different from furin, although the possibility exists that monkey furin does not share high homology with human furin. We anticipated that COS-1 cells may have PACE4. Indeed, using primers corresponding to human PACE4, a cDNA fragment of predicted size was detected by PCR (data not shown), suggesting that PACE4 exists in COS-1 cells and may be involved in processing. Further study is needed to confirm that this endopeptidase is responsible for processing of the fusion protein at the cleavage site with both P4 and P6 Arg residues.

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