BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 89–96 (1996)
Article No. 0017

Missense Mutations Associated with Familial Alzheimer's Disease in Sweden Lead to the Production of the Amyloid Peptide without Internalization of Its Precursor

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Received November 13, 1995

Production of soluble amyloid peptide precursor (APP) and amyloid peptide (Aβ) was measured in CHO cells transfected by the wild-type APP 695 cDNA sequence or by the same sequence carrying missense mutations associated with familial Alzheimer's disease in Sweden. Deletion of the C-terminal domain of the protein corresponding to residues 654 to 695 of APP695 not only inhibited very significantly the internalization of APP at 37°C, but also led to the secretion of an uncleaved APP in the culture medium of CHO cells. This deletion did not affect Aβ production from the Swedish APP but was able to inhibit the production of the wild-type APP. These results demonstrate that, in CHO cells, the internalization of the wild-type APP is needed for Aβ production, while the production of the amyloid peptide from Swedish APP is independent of the internalization process.

The neuropathology of Alzheimer's disease is characterized by extracellular deposits of fibrillar amyloid peptide in the brain parenchyma and cerebrovasculature. The 39–43 amino acid amyloid peptide, also named Aβ, results from the proteolytic cleavage of a larger precursor, the amyloid peptide precursor (APP) (1). Various cellular processing pathways are implicated in the metabolism of APP. The constitutive secretory pathway of APP delivers some of the precursor to the cell surface, and proteolytic cleavage of the transmembrane protein releases the amino terminus of the molecule from the cell (2). APP is cleaved in the secretory pathway by a proteolytic activity defined as α-secretase. The cleavage of APP by α-secretase, leads to the secretion of a large soluble ectodomain of the protein and the retention of a small C-terminal fragment within the membrane. Since the α-secretase cleaves within the amyloid peptide (3), this secretion process precludes the production of Aβ. An alternative proteolytic pathway has been observed which leads to the secretion of Aβ by cells in culture. Two enzymes referred as β- and γ-secretase cleave the amyloid peptide from its precursor, and these cleavages result in the release of Aβ into the culture medium. Recent reports describe that soluble Aβ can be detected *in vitro* in the extracellular medium from a variety of cultured mammalian cells (4–6) as well as *in vivo* in the human cerebrospinal fluid (7). The discovery of pathogenic mutations in the APP gene (8–12) provides strong evidence for the hypothesis that APP metabolism leading to altered Aβ production or deposition is an early event in the etiology of Alzheimer's disease. Here, we compared the metabolism of APP in transfected CHO cells which express either the wild-type APP or APP which harbors missense mutations associated with familial Alzheimer's disease in Sweden.

MATERIALS AND METHODS

Plasmid construction and DNA mutagenesis. The full-length cDNA of the human APP containing 695 amino acids (APP695) was cloned in the SmaI-SalI restriction sites of the pSVK3 expression vector (pSVAPPwt). To generate a plasmid containing the APP sequence that harbors the Swedish double mutation (KM-NL) upstream of the Aβ sequence, a DNA fragment was generated by polymerase chain reaction using oligonucleotide A (AGATCTCTGAAGTGAATCTGGATGC)

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0006-291X/96 $12.00

which encodes a region upstream the transmembrane domain, includes a BgIII restriction site, and contains two base transversions (G to T, A to C) at codons 595 and 596 of APP 695, and oligonucleotide B (GTCGACCTAGTTCTGCATC-TGCTCAAAGAACTT) complementary to the sequence encoding residues 688 to 695 of APP695, and containing both a stop codon and a Sal I restriction site. The amplified fragment was digested by BgIII and Sall, gel purified and subcloned in the pSVAPPwt to generate pSVAPPsw. The intracellular C-terminal domain of APP was deleted by the introduction of a stop codon after Y 653 of APP 695. Therefore, a DNA fragment was generated by polymerase chain reaction using oligonucleotide C (AACGAAGTTGAGCCTGTTGATG) encoding residues 560 to 567 of APP 695, and oligonucleotide D (GTCGACCTAGTACTGTTTCTTCTTCAGC) complementary to residues 648 to 653 of APP 695, and containing a stop codon as well as a SaII restriction site. The amplified DNA fragment was digested by BgIII and SalI, and the purified fragment was cloned in the pSVAPPwt to generate pSVAPPwt△C. A similar approach was used to generate pSVAPPsw△C, with oligonucleotides A and C.

Establishment of CHO cell lines expressing the human APP. CHO cells were routinely cultured in F12 medium (Gibco) containing 10% FCS (Gibco) and were cotransfected with 10 μg of the different pSVAPP expression plasmids and 1 μg pSV2neo using the lipofectamine method as recommended by the manufacturer (Gibco-BRL). Stable lines were selected by exposure to 0.5 mg/ml of geneticin (G418 from Gibco). After serial dilutions of the CHO transfected cells, individual clones showing high expression of APP by immunoblotting analysis were retained.

Internalization of APP. The internalization of APP was measured using the radiolabeled 22C11 monoclonal antibody. The 22C11 monoclonal antibody was purified by protein A sepharose chromatography, and labeled by ^{125}I iodine (Amersham), to a specific radioactivity of 400 cpm/ng of protein. Transfected CHO cells expressing either the full-length (APPwt) or the truncated APP (APPwt△C) were incubated for 1 h at 0°C in their culture medium containing 1 nM ^{125}I-22C11 antibody. After 3 washes with the culture medium, the cells were incubated twice for 5 min in acidic PBS (pH 2.5). In these experimental conditions, all the radiolabeled 22C11 antibody was removed from the cell surface. The reincubation of the cells at 37°C before the acidic washes allowed to measure the internalization of APP, since in these experimental conditions, both APP and 22C11 monoclonal antibody were internalised and the radioactivity became resistant to acidic washes.

SDS–PAGE and immunoblotting. A post nuclear supernatant of transfected CHO cells was prepared after homogenization of the cells using a Dounce homogenizer, and centrifugation at 300g. The supernatant was used to prepare a membrane fraction by centrifugation at 100 000g for 30 min as previously described (13). Proteins from CHO cells and from their culture medium were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane for 45 min at 5 mA/cm^2. The filter was blocked for 1h with 5% non-fat dry milk in TBS (50 mM Tris-HCl, 150 mM NaCl, 0.5% Tween 20, pH 8.1) and incubated overnight at room temperature with the primary antibody. Membranes were then incubated with a biotinylated goat anti-mouse antibody followed by a streptavidin-alkaline phosphate complex. The alkaline phosphate activity was detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The primary antibody used to detect APP was the monoclonal 3H5 antibody at a dilution of 1/1000 of the ascite fluid. This antibody is raised against the extracellular anionic domain of APP (14). For the detection of the C-terminal domain of APP, the 4G8 monoclonal antibody was used at a 1/5000 dilution of the ascite fluid (15). This antibody is raised against the amino acids 17–24 of Aβ.

Metabolic labeling and immunoprecipitation. For metabolic labeling, CHO cells were incubated overnight in 5 ml of culture medium containing 70 μCi [^{35}S]-methionine (Amersham). Cells and culture media were then harvested and used for immunoprecipitation experiments, performed in a solubilisation buffer (25 mM Tris-HCl, 0.5% Triton X100, 0.5% NP 40, pH 7.5), as previously described (16). The soluble cellular extracts or the culture media were first incubated for 1 hour in the presence of 20 μl of protein A sepharose precoated with rabbit anti-mouse IgG. After centrifugation, supernatant was recovered and incubated overnight at 4°C with the 22C11 monoclonal antibody raised against the N-terminal domain of APP, and 30 μl of protein A sepharose (Pharmacia) precoated with rabbit anti-mouse IgG. After centrifugation, the pellet was first washed twice with 10 mM Tris-HCl, 150 mM NaCl, 0.2% NP 40, 2 mM EDTA, pH 7.5, then twice with 10 mM Tris-HCl, 500 mM NaCl, 0.2% NP 40, 2 mM EDTA, pH 7.5, and then finally once with 10 mM Tris-HCl pH 7.5. The immunoprecipitate was resuspended in Laemmli sample buffer and analyzed in SDS-PAGE. The Aβ peptide was immunoprecipitated using the SGY2134 antibody as described by Shoji et al. (16), and the immunoprecipitate was analyzed on 10–16% Tris-Tricine SDS-PAGE. For both immunoprecipitations, the gels were dried and autoradiographed.

RESULTS

Production of soluble APP and Aβ by transfected CHO cells. CHO cells were stably transfected with the pSVAPPwt vector containing the human APP695 cDNA sequence, and individual clones were further characterized by immunoblotting analysis of secreted proteins using the 3H5 antibody that recognizes the anionic extracellular domain of APP (14). As shown in figure 1A, soluble APP is detected in the culture medium of transfected CHO cells (lane 2), although the 3H5 antibody does not recognize any proteins in the culture medium of untransfected cells (lane 1). This soluble APP expressed following transfection is not recognized by the 4G8 antibody raised against the amino

acids 17–24 of Aβ (Fig. 1A, lane 3). These results indicate that soluble APP produced by transfected CHO cells does not contain residues 17–24 of Aβ, consistent with the α-secretase cleavage of APP that occurs at residue 15 of Aβ (3). Concomitant with the secretion of APP by α-secretase cleavage, the production of Aβ was evaluated after metabolic labeling of CHO cells with [^{35}S]-methionine and immunoprecipitation with specific antibodies. The soluble form of APP was immunoprecipitated from the culture medium by the 22C11 monoclonal antibody (Fig. 1B) and the Aβ peptide was immunoprecipitated by the SGY2134 antibody (Fig. 1C).

Inhibition of APP internalization by deletion of the C-terminal domain of the protein. The deletion of the C-terminal domain of APP has been previously demonstrated to inhibit APP internalization (17,18). APPwt△C was generated by introduction of a stop codon after Y 653 of APP 695 (Fig. 2A). CHO cells expressing APPwt were incubated for 1h at 0°C in the presence of 1 nM of the ^{125}I-22C11 anti-APP monoclonal antibody. Following 3 washes with culture medium, the cells were dissolved in sodium deoxycholate, counted, and the protein content was measured using the Bradford's method. The binding of ^{125}I-22C11 corresponded to 619 ± 41 cpm/mg protein (n = 6). After 30 min reincubation at 37°C, 47.3% of this radioactivity was resistent to 2 acidic washes, and after 1 h incubation at 37°C, 73.6 ± 3.3% (n = 3) of the radioactivity was internalized (Fig. 2B). When the same experiment was performed with CHO cells expressing APPwt△C, the binding of ^{125}I-22C11 following 1 h incubation at 0°C corresponded to 411 ± 19 cpm/mg protein

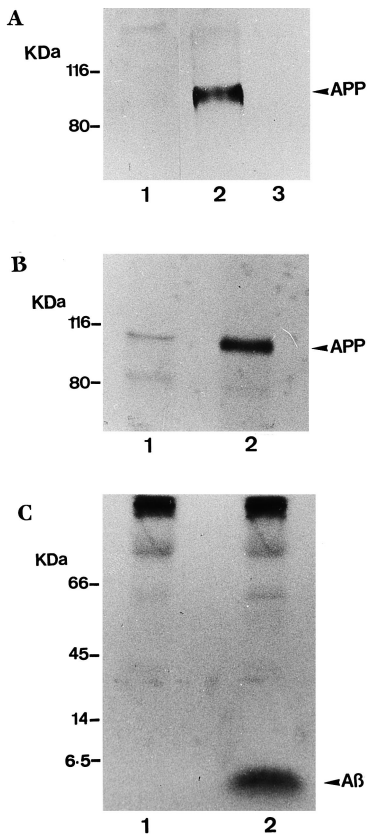

FIG. 1. Expression of APP and Aβ in CHO cells. The culture medium of control (1) or transfected cells (2,3) was analyzed in immunoblotting (A) with the 3H5 (1,2) or the 4G8 (3) monoclonal antibodies. After metabolic labeling, the culture medium of control (1) and transfected cells (2) was immunoprecipitated with the 22C11 monoclonal antibody (B) or the SGY2134 antibody (C).

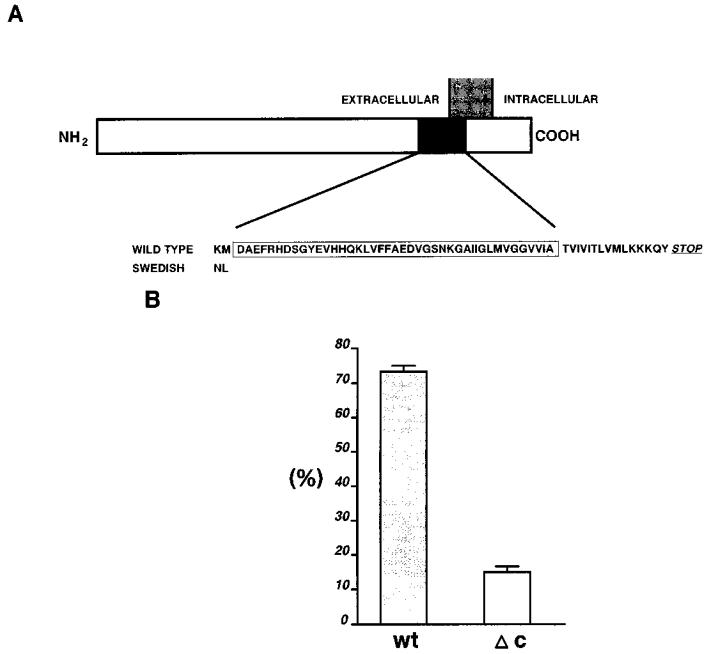

FIG. 2. Deletion of the C-terminal domain of APP inhibits its internalization. A stop codon was introduced after Y653 of APP695wt or APP695sw (A). After binding of the ^{125}I-22C11 anti-APP monoclonal antibody to the membrane APP at 0°C, internalization of APPwt (wt) or APP△C (△C) was measured after a 1h reincubation of transfected cells at 37°C (B).

(n = 3). During the reincubation at 37°C, only 11.5% ± 1.7% (n = 3) and 16.5 ± 2.7% (n = 3) of the radioactivity was internalized during a 30 min or a 1h incubation, respectively (Fig. 2B).

The amyloid peptide is produced during secretion of Swedish APP. When CHO cells were transfected with the pSV695wt△C or pSV695sw△C constructs, soluble APP produced in the extracellular medium was recognized by both the 3H5 and 4G8 antibodies (Fig. 3A). Since the 4G8 antibody is raised against the amino acids 17 to 24 of the Aβ peptide, the soluble APP produced by transfection of the pSVAPP△C constructs is not cleaved by α-secretase, at amino acid 15 of the Aβ peptide. Fifty μg of proteins isolated from a membrane fraction of CHO cells transfected by pSVAPPwt or pSVAPPwt△C were analysed in immunoblotting with the 3H5 monoclonal antibody. The results presented in figure 3B indicate that APP was detected in cells expressing the wild-type sequence (lane 1). In contrast, only a faint band of lower molecular weight was detected

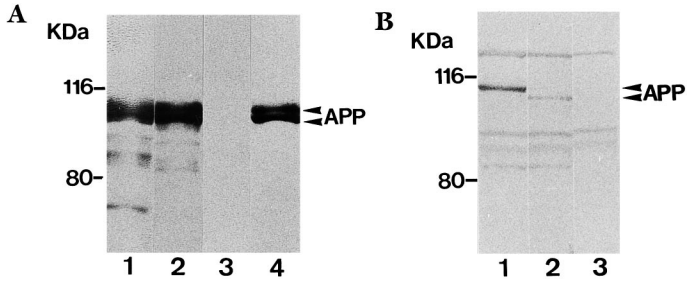

FIG. 3. Deletion of the C-terminal domain of APP leads to the secretion of uncleaved APP in the extracellular medium. The culture medium (A) of CHO cells expressing APPwt (1,3) or APP△C (2,4) was analysed in immunoblotting with the 3H5 (1,2) or the 4G8 (3,4) monoclonal antibodies. A membrane fraction (B) of the same transfected cells expressing APPwt (1) or APP△C (2) was analysed in immunoblotting with the 3H5 monoclonal antibody (1,2); the nonspecific bands revealed by the secondary antibody are shown in lane 3.

in the membrane fraction of pSVAPPwt△C transfected cells (Fig. 3B, lane 2), although these cells produce high amounts of extracellular soluble APP (Fig. 4A). From these results, it is concluded that, in CHO cells expressing APP deleted from the C-terminal domain corresponding to residues 654 to 695 of APP695, part of APP is not firmly anchored in the membrane at 37°C, and is secreted without cleavage by α-secretase. The production of the amyloid peptide was analysed after transfection of CHO cells with pSVAPPwt△C or pSVAPPsw△C. The results presented in figure 4A indicate that the deletion of the C-terminal domain of APP, leading to the secretion of uncleaved protein, precludes the production of Aβ from the wild-type APP. On the contrary, transfected cells which express APPsw△C produce Aβ like the pSVAPPsw transfected cells (Fig. 4B). All together, these results clearly demonstrate that Aβ can be produced from APPsw by a mechanism independent of the internalization process.

DISCUSSION

The identification of APP mutations genetically linked to familial Alzheimer's disease and the findings that these mutations substantially alters APP processing (19,20) strongly support that APP metabolism plays a role in the etiology of the disease. Two metabolic pathways of APP have been described. Cultured cells secrete C-terminal truncated APP into the medium. These proteins are derived from the transmembrane APP isoforms by proteolysis and have lost the cytoplasmic and the transmembrane domains. Amino acid analyses showed that a constitutive processing event, mediated by an unknown protease activity called α-secretase, cleaves APP within the Aβ peptide, thus preventing Aβ deposition (3). Since α-secretase activity is not inhibited by exogenously added proteinase inhibitors of different specificity (21), the enzyme is apparently located intracellularly. No inhibition of α-secretase by lysosomotropic agents but by methylamine suggests that APP cleavage occurs in a late compartment of the default secretion pathway (22).

In addition to this secretory pathway, C-terminal fragments of APP were identified in cultured cells (23) or in brain tissue (24) that are long enough to contain the full-length Aβ sequence and therefore are amyloidogenic. Cultured cells that express APP are also able to release Aβ in the extracellular medium (4–6). The C-terminal cleavage generating the Aβ peptide is performed by an unknown protease called γ-secretase. The γ-secretase cleavage occurs somewhere between amino acid 39 and the end of the Aβ sequence. The N-terminal cleavage generating Aβ appears to be mediated by a highly sequence-dependent protease named β-secretase (25), distinct from α-secretase which cleaves APP in a sequence independent manner.

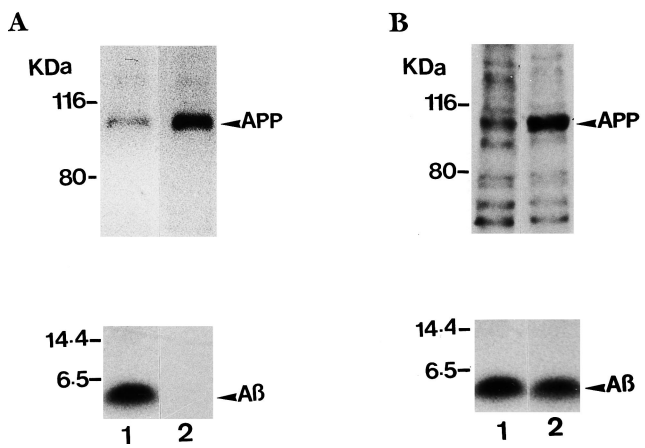

FIG. 4. The production of Aβ from APPsw is independent of the internalization process. The culture medium of CHO cells expressing APPwt (A,1), APPwt△C (A,2), APPsw (B,1) or APPsw△C (A,2), APPsw (B,1) or APPsw△C (B,2) was immunoprecipitated with the 22C11 monoclonal antibody (upper panel) or the SGY2134 antibody (lower panel).

The cellular mechanisms that produce Aβ were analyzed by treatment of cultured cells with a variety of agents that interfere with cellular processing pathways. The complete inhibition of Aβ generation by brefeldin A, which causes a redistribution of Golgi into endoplasmic reticulum, suggests that transport of APP through the Golgi is a prerequisite for Aβ production (26). Aβ formation is not affected by leupeptin which inhibits lysosomal proteases (5,6,26). However, agents that interfere with pH in acidic compartments like NH_4Cl and chloroquine inhibit Aβ production (5,26), suggesting that an acidic compartment is necessary for Aβ generation. From these results it was proposed that the production of Aβ could take place within the acidic compartments of late Golgi or transport vesicles derived from a reinternalization pathway carrying APP or fragments thereof (26,27). Recently, radiolabeled Aβ was recovered in the extracellular medium following selective cell surface radioiodination of cultured cells, indicating that cell surface APP is a direct precursor of Aβ (17). In addition, deletion of the cytoplasmic domain of APP containing a consensus motive for coated-pit mediated internalization (NPTY), resulted in reduced APP internalization and diminished Aβ release (17,18). Inhibition of endocytic clathrin-coated pit formation can be achieved by intracellular potassium depletion. In CHO cells, it has been previously demonstrated that potassium depletion inhibits coated-pit mediated endocytosis of transferrin and prevents the production of Aβ (17). However, potassium depletion does not significantly affect the production of soluble extracellular APP (17). These results indicate that the cellular trafficking of APP plays a key role in the choice between the two possible catabolic pathways of the protein. Whereas the secretion pathway produces non amyloidogenic soluble APP, the endocytic pathway leads to Aβ production. Recently, several mutations in genes from chromosomes 14 and 1 have been demonstrated to be associated with familial Alzheimer's disease (28,29). It is now important to study whether the corresponding proteins are involved in the cellular metabolism of APP.

Missense mutations associated with familial Alzheimer's disease have also been mapped to the APP gene. The double mutation at the Aβ N-terminus, which is associated with familial Alzheimer's disease in Sweden, causes a 3 to 8 fold increase of Aβ production (19,20). The metabolism of the Swedish APP has been studied in various cell types. In Madin-Darby canine kidney cells, about 90% of the wild-type APP is secreted basolaterally following α-secretase cleavage, while soluble derivatives of Swedish APP cleaved at the β-secretase site are secreted into the apical compartment (30). In 293 cells, the deletion of the C-terminal domain of APP corresponding to residues 648 to 695 of APP695 has a clear influence on β-secretase cleavage of wild-type APP, since peptides starting at Arg-5 instead of Asp-1 are generated (25). The same deletion has no effect on the β-secretase cleavage of Swedish APP (25). Recently, the vacuolar H^+-ATPase inhibitor bafilomycin A1 was demonstrated to strongly increase the production of Aβ beginning at Ile-6, Val-3 and Phe-4, by kidney 293 cells which express wild-type APP, while the production of Aβ starting at Asp-1 was decreased (31). Kidney 293 cells transfected with the Swedish APP sequence and treated with bafilomycin A1 did not produce these alternative Aβ peptides and bafilomycin A1 treatment resulted in a decrease of Aβ starting at Asp-1 (31). These results suggest that different enzymes with the characteristics of β-secretase-type proteases are able to create the various N-terminini of Aβ from the wild-type APP, while kidney 293 cells expressing the Swedish APP exclusively secrete Aβ peptide beginning at Asp1, even after deletion of C-terminal domain of APP (25). In CHO cells, both the wild-type and the Swedish APP produce Aβ beginning at Asp-1, and bafilomycin AJ treatment does not result in an increase of Aβ production (31).

In this paper, we demonstrate that in CHO cells expressing APP deleted from the C-terminal domain corresponding to residues 654 to 695 of APP695, cellular APP can bind an anti-APP antibody at 0°C. At 37°C however, most of the truncated APP is not internalized but is released in the extracellular medium without cleavage by α-secretase. Such a leakage of truncated APP from the membrane was already observed in kidney 293 cells (25). However, the release of uncleaved APP in the extracellular medium, and the absence of Aβ production from APP was only observed

when kidney 293 cells were transfected with an APP sequence containing a larger deletion corresponding to residues 637 to 695 of APP695 (25). In our study, CHO cells, which secrete an uncleaved Swedish APP, produce Aβ in amounts very similar to CHO cells expressing the full-length Swedish APP. On the contrary, CHO cells which secrete an uncleaved wild-type APP are unable to produce Aβ. These results clearly demonstrate that the production of Aβ from wild-type and Swedish APP occurs in two different cellular compartments, although Aβ produced by CHO cells from wild-type or Swedish APP begins at Asp-1 (31). Internalization of the wild-type APP is needed for Aβ production, while the production of the amyloid peptide from Swedish APP is independent of the internalization process.

ACKNOWLEDGMENTS

We thank S. Younkin and K.S. Kim for providing the antibodies SGY2134 and 4G8, respectively. We also thank B. Tasiaux-Doumont for excellent technical assistance. This work was supported by grants from the Fund for Medical Scientific Research (Belgium), the Queen Elisabeth Medical Foundation (Belgium), and the Bioavenir program with the participation of the French ministry of research and the French ministry of industry. J.N.O. is Research Associate of the National Fund for Scientific Research (Belgium).

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