

# A presenilin-1/ $\gamma$ -secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions

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**E-cadherin controls a wide array of cellular behaviors including cell–cell adhesion, differentiation and tissue development. Here we show that presenilin-1 (PS1), a protein involved in Alzheimer’s disease, controls a  $\gamma$ -secretase-like cleavage of E-cadherin. This cleavage is stimulated by apoptosis or calcium influx and occurs between human E-cadherin residues Leu731 and Arg732 at the membrane–cytoplasm interface. The PS1/ $\gamma$ -secretase system cleaves both the full-length E-cadherin and a transmembrane C-terminal fragment, derived from a metalloproteinase cleavage after the E-cadherin ectodomain residue Pro700. The PS1/ $\gamma$ -secretase cleavage dissociates E-cadherins,  $\beta$ -catenin and  $\alpha$ -catenin from the cytoskeleton, thus promoting dis-assembly of the E-cadherin–catenin adhesion complex. Furthermore, this cleavage releases the cytoplasmic E-cadherin to the cytosol and increases the levels of soluble  $\beta$ - and  $\alpha$ -catenins. Thus, the PS1/ $\gamma$ -secretase system stimulates disassembly of the E-cadherin–catenin complex and increases the cytosolic pool of  $\beta$ -catenin, a key regulator of the Wnt signaling pathway.**

**Keywords:** Alzheimer’s disease/ $\beta$ -catenin/E-cadherin/presenilin-1/ $\gamma$ -secretase

## Introduction

Classic cadherins, including epithelial (E)- and neural (N)-cadherins, are major cell–cell adhesion receptors involved in the development, maintenance and function of most tissues, including the nervous system, epithelia and endothelia (Steinberg and McNutt, 1999; McNeill, 2000; Tepass *et al.*, 2000). In addition, cadherins play important roles in cell signaling, proliferation and differentiation (Steinberg and McNutt, 1999; Tepass *et al.*, 2000). In cadherin-based adherens junctions (CAJs), the extracellular domains of transmembrane cadherins promote cell–cell adhesion by engaging in Ca<sup>2+</sup>-dependent homophilic interactions, while the cytoplasmic domains are linked to the actin cytoskeleton via  $\alpha$ -

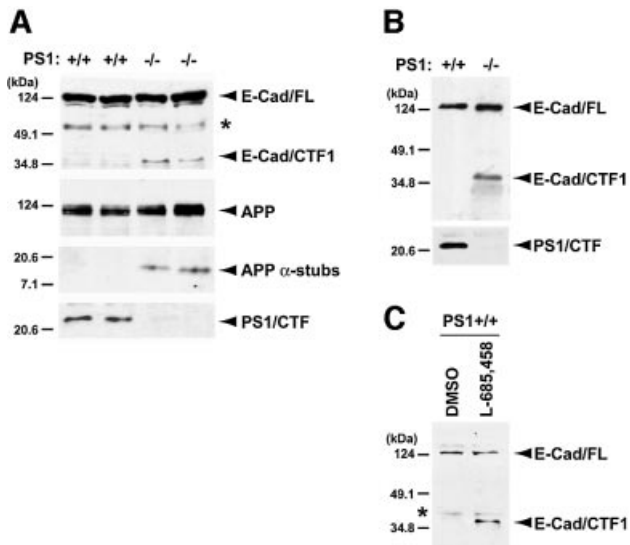
and  $\beta$ -catenins (Steinberg and McNutt, 1999; Gumbiner, 2000). Post-translational regulation of cadherin adhesive activities, including proteolytic processing of cadherins and disassembly of CAJs, plays crucial roles in rapid changes in cell adhesion, signaling and apoptosis (Herren *et al.*, 1998; Gumbiner, 2000; Vallorosi *et al.*, 2000; Steinhusen *et al.*, 2001), but the molecular mechanisms involved in cadherin processing and CAJ disassembly remain mostly unknown.

PS1 is a polytopic transmembrane protein involved in most cases of early-onset familial Alzheimer’s disease (FAD). Cellular PS1 is cleaved to yield an N-terminal (PS1/NTF) and a C-terminal (PS1/CTF) fragment. Following cleavage, the resultant PS1 fragments form a stable 1:1 heterodimer (Thinakaran *et al.*, 1996; Podlisny *et al.*, 1997) that binds to the cytoplasmic juxtamembrane region of E-cadherin (Baki *et al.*, 2001). PS1 is found in the endoplasmic reticulum (ER)–Golgi system and the neuronal somatodendritic compartment (Cook *et al.*, 1996; Elder *et al.*, 1996; Kovacs *et al.*, 1996), but upon formation of cell–cell contacts PS1 concentrates at intercellular sites at the cell surface where it forms complexes with the CAJ (Georgakopoulos *et al.*, 1999). In addition to E-cadherin, PS1 forms complexes with N-cadherin and it has been localized at synaptic sites (Georgakopoulos *et al.*, 1999). Recently it was reported that PS1 regulates a  $\gamma$ -secretase cleavage of both APP and Notch receptor and stimulates A $\beta$  production (De Strooper *et al.*, 1999; Selkoe, 2001). Here we show that apoptosis or Ca<sup>2+</sup> influx stimulates a PS1/ $\gamma$ -secretase cleavage of E-cadherin. This cleavage results in the release of the cytoplasmic sequence of E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin to the soluble cytosol, thus facilitating disassembly of CAJs. In contrast to APP and Notch, however, both of which are cleaved within their transmembrane sequence (Schroeter *et al.*, 1998; Gu *et al.*, 2001; Sastre *et al.*, 2001; Yu *et al.*, 2001), the PS1/ $\gamma$ -secretase cleavage of E-cadherin takes place at the membrane–cytosol interface.

## Results

### A PS1/ $\gamma$ -secretase activity controls E-cadherin processing

We used extracts from PS1 knock-out mouse embryos (PS1<sup>−/−</sup>) (Baki *et al.*, 2001) to investigate whether PS1 plays any role in E-cadherin processing. PS1<sup>−/−</sup> embryos had significantly higher amounts of a 38 kDa peptide that contained the cytoplasmic sequence of E-cadherin (E-Cad/CTF1) than did wild-type (PS1<sup>+/+</sup>) embryos, even though all embryos had similar levels of the full-length E-cadherin (Figure 1A, upper panel). As expected (De Strooper *et al.*, 1999), PS1<sup>−/−</sup> embryos also contained increased levels of APP  $\alpha$ -stubs (Figure 1A, middle panels). E-Cad/CTF1



**Fig. 1.** A PS1-mediated  $\gamma$ -secretase activity controls E-cadherin processing. (A) Extracts from PS1<sup>+/+</sup> or PS1<sup>-/-</sup> mouse embryos were probed on western blots with either anti-cytoplasmic E-cadherin C36 (upper panel) or anti-cytoplasmic APP R1 (middle panels) antibodies. E-Cad/FL denotes full-length E-cadherin. The asterisk identifies mouse IgGs. Lower panel: extract probed with anti-PS1/CTF antibody 33B10. (B) Extracts from E-cadherin-transfected PS1<sup>+/+</sup> or PS1<sup>-/-</sup> mouse fibroblasts were probed with anti-E-cadherin C36 (upper panel) or 33B10 (lower panel) antibodies. (C) PS1<sup>+/+</sup> fibroblasts were treated for 6 h either with the  $\gamma$ -secretase inhibitor L-685,458 (0.5  $\mu$ M) or with dimethylsulfoxide. Extracts from these cell cultures were then probed with anti-E-cadherin C36. The asterisk indicates a non-specific band.

accumulates in a fibroblast cell line that was derived from PS1<sup>-/-</sup> mice (Baki *et al.*, 2001) compared with a cell line from PS1<sup>+/+</sup> mice, even though both cell lines express comparable amounts of transfected full-length E-cadherin (Figure 1B). The accumulation of E-Cad/CTF1 in PS1<sup>-/-</sup> cells under conditions of constant levels of the full-length protein suggests that a PS1-mediated activity may control metabolism of E-Cad/CTF1. Indeed, treatment of E-cadherin-transfected PS1<sup>+/+</sup> fibroblasts with the selective  $\gamma$ -secretase inhibitor L-685,458 (Li *et al.*, 2000; Shearman *et al.*, 2000) increased cellular E-Cad/CTF1 compared with non-treated controls (Figure 1C), indicating that the PS1-associated  $\gamma$ -secretase activity (Esler and Wolfe, 2001) may be involved in the metabolism of E-Cad/CTF1.

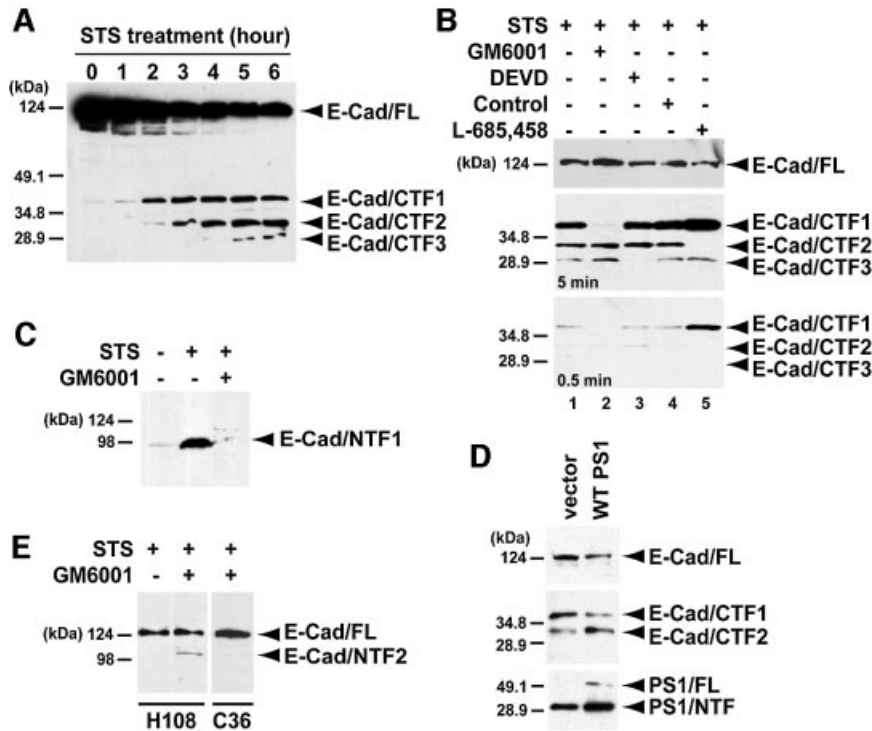
Although our data suggested that peptide E-Cad/CTF1 is processed further by a PS1/ $\gamma$ -secretase-dependent cleavage, we were unable to detect any E-cadherin metabolites resulting from this activity either in embryos or in PS1<sup>+/+</sup> fibroblasts. The apparent molecular weight and immunoreactivity of E-Cad/CTF1, however, suggested that this fragment derives from a matrix metalloproteinase (MMP) cleavage of the E-cadherin ectodomain. Since this cleavage is stimulated by apoptosis (Steinhusen *et al.*, 2001), we asked whether the levels of the PS1/ $\gamma$ -secretase cleavage product of E-cadherin might increase in apoptotic conditions. Human epithelial cell line A431 that expresses high levels of endogenous E-cadherin and undergoes apoptosis under staurosporine (STS) treatment (Steinhusen *et al.*, 2001) was used as a model. STS treatment of this cell line resulted in a time-dependent

production of three E-cadherin C-terminal fragments migrating at 38, 33 and 29 kDa, respectively (Figure 2A). Production of the 38 kDa fragment is inhibited by the MMP inhibitor GM6001 (Galardy *et al.*, 1994), indicating that this fragment, which has immunoreactivity and apparent molecular mass identical to E-Cad/CTF1 (Figure 1), is derived from an MMP cleavage of E-cadherin (Figure 2B, middle panel, lanes 1 and 2). STS also increased a secreted 95 kDa fragment detected with E-cadherin ectodomain antibody H108 (Figure 3). This fragment (termed E-Cad/NTF1, Figure 2C) does not react with antibodies against cytoplasmic E-cadherin (not shown) and, like E-Cad/CTF1, it is also inhibited by GM6001 (Figure 2C), suggesting that E-Cad/NTF1 is the secreted counterpart of E-Cad/CTF1 (Figure 3). The 29 kDa fragment (E-Cad/CTF3) is inhibited by the specific caspase-3 inhibitor Z-DEVD-FMK (Figure 2B, middle panel, lanes 1 and 3), indicating that it is produced by an apoptosis-stimulated caspase-3 cleavage of E-cadherin (Steinhusen *et al.*, 2001).

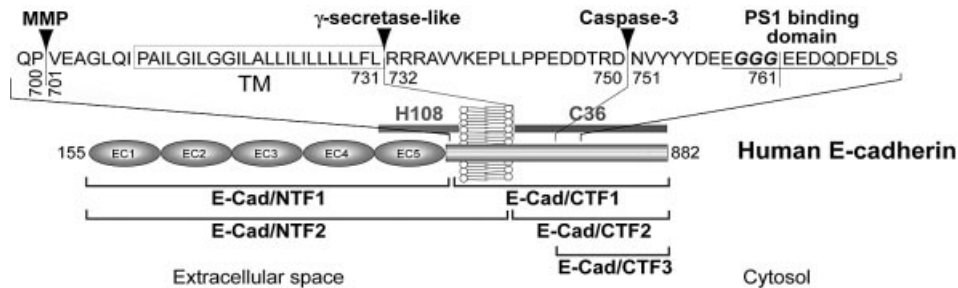
The  $\gamma$ -secretase inhibitor L-685,458 completely blocked production of the 33 kDa cadherin fragment (E-Cad/CTF2; Figure 2B, middle panel, lanes 1, 4 and 5), indicating that this fragment is produced by a  $\gamma$ -secretase-like cleavage of E-cadherin. Inhibition of E-Cad/CTF2 by L-685,458 correlates with an increase in E-Cad/CTF1 (Figure 2B, lower panels, lanes 4 and 5), suggesting that the former peptide derives from the latter by a  $\gamma$ -secretase-like activity. Accordingly, overexpression of PS1 in cell line HEK293 increased E-Cad/CTF2 and decreased E-Cad/CTF1 (Figure 2D). That E-Cad/CTF2 is produced even when E-Cad/CTF1 is inhibited (Figure 2B, lane 2) suggests that the PS1/ $\gamma$ -secretase-dependent E-Cad/CTF2 can also be derived from full-length E-cadherin. In agreement with this suggestion, a 100 kDa E-cadherin fragment recognized by antibody H108 but not by anti-cytoplasmic E-cadherin antibody C36 (see Figure 3 for sequences recognized by these antibodies) was detected in cell extracts of GM6001-treated A431 cultures (Figure 2E), suggesting that this fragment (E-Cad/NTF2) is the N-terminal counterpart of E-Cad/CTF2 (see also Figure 3). In addition to A431, two other cell lines, SW480 and LNCaP, also produced the PS1/ $\gamma$ -secretase fragment E-Cad/CTF2 under Ca<sup>2+</sup> influx conditions (data not shown), indicating that this is a general mechanism of E-cadherin processing.

#### Identification of the PS1/ $\gamma$ -secretase and MMP cleavage sites of E-cadherin

E-Cad/CTF1 and E-Cad/CTF2 were affinity purified from STS-treated A431 cells. Antibody H108 against E-cadherin sequence 600–707 (numbering according to the full-length unprocessed human E-cadherin) reacted with secreted E-Cad/NTF1 (Figure 2C) but not with cellular E-Cad/CTF1 (not shown), suggesting that the MMP cleavage of E-cadherin occurs closer to the extracytoplasmic face of the plasma membrane than previously reported (Ito *et al.*, 1999). Indeed, Edman sequencing of E-Cad/CTF1 through 14 cycles showed the following major sequence: VEAGLQIPAILGIL. This is a unique sequence corresponding to human E-cadherin residues 701–714. The N-terminus of this sequence is located seven residues upstream of the transmembrane



**Fig. 2.** A PS1-mediated  $\gamma$ -secretase activity cleaves E-cadherin. (A) A431 cells were treated for the indicated times with 1  $\mu$ M of staurosporine (STS) to induce apoptosis (Steinhusen *et al.*, 2001), solubilized in RIPA and blotted with anti-E-cadherin C36 antibody. (B) A431 cells were pre-incubated for 30 min in the absence (-) or presence (+) of GM6001 (2.5  $\mu$ M), Z-DEVD-FMK (DEVD, 50  $\mu$ M), an inactive analogue of L-685,458 (Control, 0.5  $\mu$ M) or L-685,458 (0.5  $\mu$ M). Cells were then treated with STS for 6 h to induce apoptosis, and cell extracts were probed with C36 antibody. Middle and lower panels: the filter was exposed to X-ray films for either 5 or 0.5 min. (C) Conditioned media (20  $\mu$ l) from A431 cells cultured in the absence (-) or presence (+) of GM6001 and treated with STS as above were probed on western blots with anti-E-cadherin ectodomain antibody H108. E-Cad/NTF1 indicates the secreted E-cadherin ectodomain. (D) Extracts from HEK293 cells stably transfected with either wild-type (WT) PS1 or vector alone were immunoprecipitated and probed with C36 antibody (upper panels). Lower panel: extracts from the above cells were probed with anti-PS1/NTF antibody R222. (E) A431 cells were pre-incubated for 30 min in the absence (-) or presence (+) of GM6001 (2.5  $\mu$ M). Cells were then treated with STS for 6 h and cell extracts were probed on western blots with either H108 (left panel) or C36 (right panel) antibodies.



**Fig. 3.** Amino acid sequence of human E-cadherin (SWISS-PROT accession No. P12830) indicating the N-termini of E-Cad/CTF1 and E-Cad/CTF2. Arrows identify the cleavage sites of MMP and PS1/ $\gamma$ -secretase. The sequence mediating E-cadherin-PS1 binding is underlined (Baki *et al.*, 2001). Caspase-3 cleavage was reported recently (Steinhusen *et al.*, 2001). EC1-5 denote the extracellular E-cadherin repeats. TM, transmembrane domain. H108 and C36 are immunogenic regions recognized by the respective antibodies.

sequence of E-cadherin (Figure 3). Mass spectrometry analysis of E-Cad/CTF1 showed no peptides upstream of the cleavage site that was determined by Edman sequencing (not shown). Thus, the 38 kDa E-Cad/CTF1 is produced by an MMP cleavage after E-cadherin residue Pro700 (Figure 3).

Edman sequencing of E-Cad/CTF2 yielded the following sequence: RRRVVVKEPLL. This is a unique sequence corresponding to human E-cadherin residues 732-742. Mass spectrometric analysis of E-Cad/CTF2

yielded E-cadherin peptides predicted from the sequencing data (not shown). These results show that the PS1-mediated  $\gamma$ -secretase cleavage of E-cadherin takes place between residues Leu731 and Arg732 at the interface of the membrane with the cytoplasm (Figure 3).

**The  $\gamma$ -secretase-mediated cleavage of E-cadherin promotes disassembly of adherens junctions**

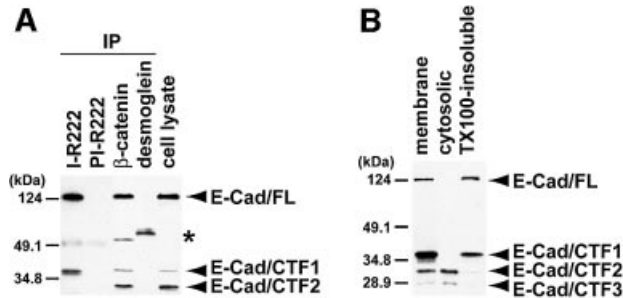
The molecular weight and immunoreactivity of the isolated peptides suggest that they contain the entire

E-cadherin cytoplasmic sequence including the  $\beta$ -catenin-binding site (Steinberg and McNutt, 1999). Co-immunoprecipitation experiments showed that the MMP cleavage product E-Cad/CTF1 binds both  $\beta$ -catenin and PS1, whereas the  $\gamma$ -secretase product E-Cad/CTF2 binds only  $\beta$ -catenin (Figure 4A). Thus, the PS1/ $\gamma$ -secretase cleavage of E-cadherin dissociates PS1 from the E-cadherin- $\beta$ -catenin complex.

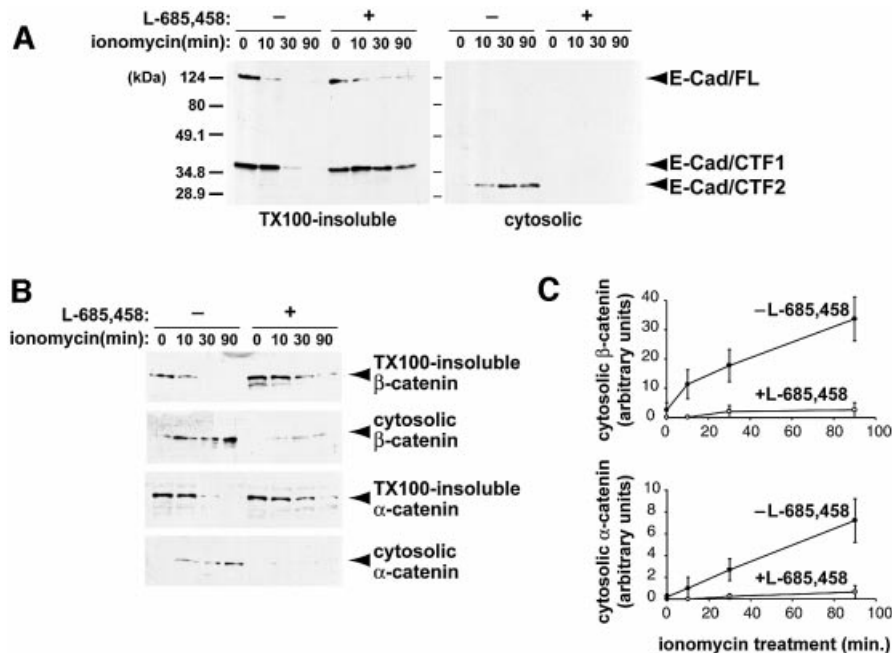
Subcellular fractionation of STS-treated A431 cells showed that full-length E-cadherin and E-Cad/CTF1

are found only in the membrane and cytoskeletal (Triton X-100-insoluble) fractions while E-Cad/CTF2 localizes in the membrane and in the soluble cytosol (Figure 4B), suggesting that the PS1/ $\gamma$ -secretase cleavage results in the solubilization of the cytoplasmic sequence of E-cadherin.

In stable cell-cell adhesion, the E-cadherin- $\beta$ -catenin complex of the CAJ is anchored to the actin cytoskeleton via  $\alpha$ -catenin, and this association is manifested by the insolubility of the complex components in Triton X-100 (Nathke *et al.*, 1994; Baki *et al.*, 2001). Induction of apoptosis or calcium influx disrupts cadherin-mediated cell-cell adhesion by cleaving cadherin and disassembling the CAJ complexes (Herren *et al.*, 1998; Ito *et al.*, 1999; Vallorosi *et al.*, 2000; Steinhilber *et al.*, 2001). To determine whether the  $\gamma$ -secretase cleavage of E-cadherin is involved in the CAJ disassembly, we induced calcium influx with ionomycin in A431 cells in the absence or presence of the  $\gamma$ -secretase inhibitor L-685,458. In the absence of this inhibitor, ionomycin induced a time-dependent decrease in the cytoskeletal (Triton X-100-insoluble) fraction of both full-length E-cadherin and E-Cad/CTF1 (Figure 5A, left panel), and this decrease correlated with a corresponding increase in soluble cytosolic E-cadherin and E-Cad/CTF1 (Figure 5A, right panel). Similarly, ionomycin decreased the cytoskeletal association (Triton X-100-insoluble fraction) of the CAJ components  $\beta$ - and  $\alpha$ -catenin with a concomitant significant increase in their soluble cytosolic levels (Figure 5B). These data indicate that ionomycin induces a time-dependent disassembly of the E-cadherin-catenin cyto-



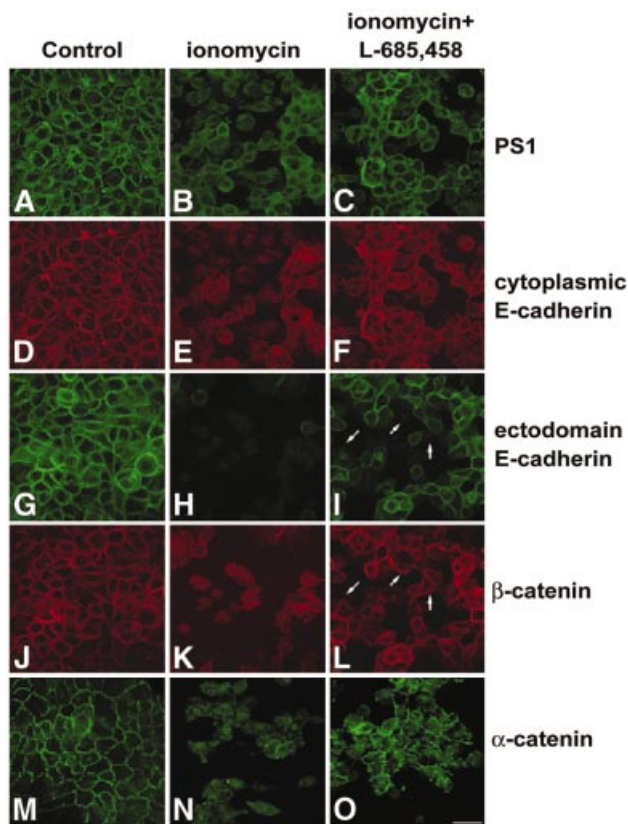
**Fig. 4.** Released E-Cad/CTF2 dissociates from PS1 but remains bound to  $\beta$ -catenin. (A) Extracts from STS-treated A431 cells were immunoprecipitated with antibodies against PS1 (I-R222), pre-immune serum (PI-R222),  $\beta$ -catenin or desmoglein, and the immunoprecipitates (IPs) obtained were probed on western blots with anti-E-cadherin antibody C36. For reference, cell lysate was also probed; the asterisk shows IgGs. (B) A431 cells treated for 6 h with STS were fractionated into membrane, soluble cytosolic and Triton X-100-insoluble (TX100-insoluble) fractions, and the fractions obtained were then probed on western blots with C36 antibody.



**Fig. 5.** A PS1/ $\gamma$ -secretase cleavage promotes disassembly of the E-cadherin-catenin adhesion complex. (A and B) A431 cell cultures were pre-incubated for 30 min in the absence (-) or presence (+) of L-685,458 and then treated for the indicated times with ionomycin (10  $\mu$ M). Cell extracts were fractionated, and the Triton X-100-insoluble and cytosolic soluble fractions were analyzed on western blots with antibodies against cytoplasmic E-cadherin C36 (A) or  $\beta$ - and  $\alpha$ -catenins (B). The immunoblots are representative of three independent experiments. (C) Signals from cytosolic  $\beta$ -catenin (upper graph) or  $\alpha$ -catenin (lower graph) obtained from ionomycin-treated cultures in the presence (+L-685,458) or absence (-L-685,458) of  $\gamma$ -secretase inhibitor L-685,458 were quantified by densitometric analysis. The graphs show the averaged immunoreactivities observed in three independent experiments.

skeletal complex resulting in increased production of the PS1/ $\gamma$ -secretase fragment E-Cad/CTF2 and in the solubilization of cytoskeletal  $\beta$ - and  $\alpha$ -catenins. L-685,458 blocked the ionomycin-induced metabolism of cytoskeletal E-Cad/CTF1 and partially inhibited degradation of the full length E-cadherin (Triton X-100 insoluble, Figure 5A, left panel) while it abolished production of soluble cytosolic E-Cad/CTF2 (Figure 5A, right panel), suggesting that L-685,458 inhibits the  $\gamma$ -secretase cleavage of both cytoskeletal full-length E-cadherin and E-Cad/CTF1. In addition, L-685,458 delayed the ionomycin-induced decrease of cytoskeletal  $\beta$ - and  $\alpha$ -catenin and inhibited their release to the soluble cytosol (Figure 5B and C). These data show that the  $\gamma$ -secretase cleavage of E-cadherin promotes dissociation of the CAJ components from the cytoskeleton and their release into the soluble cytosol.

The PS1/ $\gamma$ -secretase role in the disassembly of CAJ was examined further using laser scanning confocal micro-



**Fig. 6.** Immunostaining and LSCM analysis of ionomycin- and L-685,458-treated A431 cultures. A431 cells were pre-incubated for 30 min in the presence or absence of L-685,458 and then treated for 45 min with ionomycin. Control cells were not treated. Following the ionomycin-induced cell-cell dissociation, the distribution of PS1, E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin in all cultures was analyzed by LSCM using the constant detector setting. Cells were double labeled either with anti-PS1/NTF antibody R222 (A–C) and anti-cytoplasmic E-cadherin antibody C36 (D–F), or with anti-ectodomain E-cadherin antibody H108 (G–I) and anti- $\beta$ -catenin antibody (J–L). Cells were also labeled for  $\alpha$ -catenin (M–O). Arrows indicate a cell population showing  $\beta$ -catenin immunoreactivity at the cell surface without ectodomain E-cadherin labeling (I and L). Scale bar = 30  $\mu$ m.

scopy (LSCM). PS1, cytoplasmic and ectodomain sequences of E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin concentrated at cell-cell contacts in confluent A431 cells (Figure 6, left panels; see also Georgakopoulos *et al.*, 1999). Ionomycin treatment disrupted cell-cell adhesion and decreased plasma membrane staining of all epitopes. PS1, cytoplasmic E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin staining became more diffuse throughout the cytoplasm (Figure 6, middle panels). In contrast, cadherin ectodomain staining was now barely detectable, suggesting a significant reduction in the cellular levels of this epitope (Figure 6H), in agreement with the ionomycin-induced cleavage and secretion of E-cadherin ectodomain (see also Ito *et al.*, 1999).

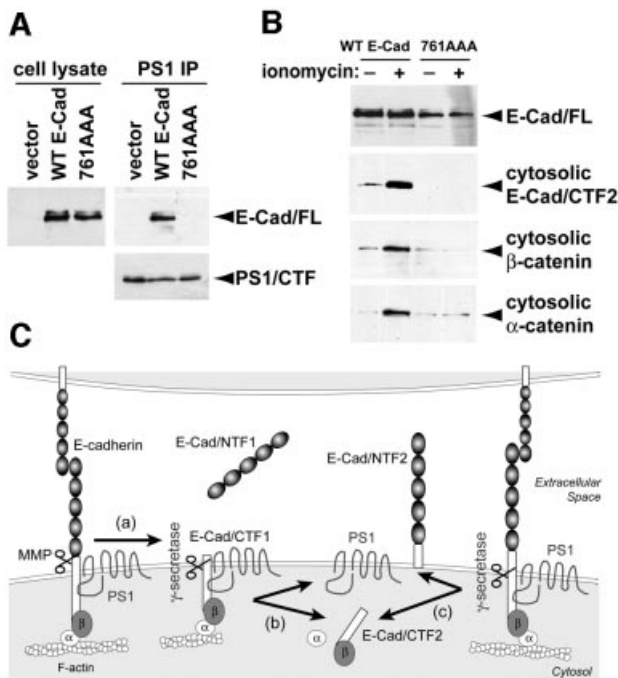
Pre-incubation with L-685,458 significantly delayed loss of cell surface staining of all epitopes (Figure 6, right panels). Ectodomain E-cadherin staining was also partially preserved at the cell surface and at cell-cell contacts (Figure 6G–I), in agreement with our finding that L-685,458 inhibits the ionomycin-induced metabolism of cytoskeletal full-length E-cadherin (Figure 5A). However, two-color immunofluorescence of L-685,458-treated cells revealed a cell population containing  $\beta$ -catenin but no ectodomain E-cadherin at the cell surface (arrows in Figure 6I and L), suggesting that these represent junctional complexes of  $\beta$ -catenin with E-Cad/CTF1 (see also Figure 4A). In L-685,458-treated cultures, there was a complete junctional overlap between staining of cytoplasmic E-cadherin and PS1 (Figure 6C and F), suggesting that cell surface PS1 remains bound to both full-length E-cadherin and E-Cad/CTF1. This observation concurs with our biochemical data showing that PS1 binds both full-length E-cadherin and E-Cad/CTF1 (Figure 4A).

#### **A cadherin mutant unable to bind PS1 is not cleaved by the PS1/ $\gamma$ -secretase activity**

To examine whether PS1 binding to E-cadherin is necessary for the PS1/ $\gamma$ -secretase cleavage, we used E-cadherin mutant GGG759-761AAA (Thoreson *et al.*, 2000). E-cadherin-negative A431D cells were transfected either with wild-type E-cadherin or with the E-cadherin mutant. In contrast to wild-type protein, mutant E-cadherin failed to bind PS1 (Figure 7A), consistent with reports that the E-cadherin sequence 760–771 (corresponding to residues 604–615 of mature processed E-cadherin) is necessary for PS1–E-cadherin binding (Baki *et al.*, 2001). Upon ionomycin treatment, cells expressing wild-type E-cadherin showed a significant increase in soluble E-Cad/CTF2,  $\beta$ -catenin and  $\alpha$ -catenin. In contrast, cells expressing mutant E-cadherin showed no ionomycin-induced increase in soluble  $\beta$ - or  $\alpha$ -catenin (Figure 7B). Furthermore, no soluble E-Cad/CTF2 was detected in mutant transfectants in either the presence or absence of ionomycin (Figure 7B). Thus, PS1 binding to E-cadherin is required for the PS1/ $\gamma$ -secretase cleavage of E-cadherin and for the release of E-Cad/CTF2,  $\beta$ -catenin and  $\alpha$ -catenin into the soluble cytosol.

## **Discussion**

Disassembly of CAJs and abrogation of cadherin-mediated cell-cell adhesion is involved in changes of cell state including differentiation, apoptosis and tumor metastasis.



**Fig. 7.** E-cadherin mutation GGG759-761AAA prevents binding to PS1 and inhibits  $\gamma$ -secretase cleavage of E-cadherin and cytosolic release of catenins. (A) Right panel: extracts from A431D cells stably transfected with vector, wild-type E-cadherin (WT E-Cad) or E-cadherin mutant GGG759-761AAA (761AAA) were immunoprecipitated with anti-PS1/NTF antibody R222 (PS1 IP) and the IPs were probed with either anti-E-cadherin antibody C36 (upper panel) or anti-PS1/CTF antibody 33B10 (lower panel). The left panel shows relative E-cadherin levels in transfectants. (B) A431D cells transfected with either wild-type E-cadherin or E-cadherin mutant 761AAA were incubated in the absence (-) or presence (+) of ionomycin for 45 min, and RIPA extracts were probed on western blots with C36 antibody (upper panel). Cytosolic fractions of the above cultures were probed on western blots with antibodies against E-cadherin (C36, second panel),  $\beta$ -catenin (third panel) or  $\alpha$ -catenin (lower panel). (C) Schematic representation of the PS1/ $\gamma$ -secretase-mediated disassembly of CAJs. An MMP-mediated proteolytic activity cleaves the extracellular domain of cytoskeletal E-cadherin and releases E-Cad/NTF1 to the extracellular medium (a). Fragment E-Cad/CTF1 containing the transmembrane and cytoplasmic sequence of E-cadherin remains bound to PS1,  $\beta$ -catenin,  $\alpha$ -catenin and the actin cytoskeleton. E-Cad/CTF1 is then cleaved by a PS1/ $\gamma$ -secretase activity at the membrane-cytosol interface to produce E-Cad/CTF2, which dissociates from both PS1 and F-actin and is released to the cytosol in a complex with  $\beta$ -catenin (b). Full-length E-cadherin bound to the cytoskeleton can also be cleaved by the PS1/ $\gamma$ -secretase activity (c). No E-Cad/CTF2- $\alpha$ -catenin complex was detected, suggesting that  $\alpha$ -catenin dissociates from E-Cad/CTF2 (unpublished observations).  $\alpha$ ,  $\alpha$ -catenin;  $\beta$ ,  $\beta$ -catenin.

For example, induction of apoptotic cell death or  $\text{Ca}^{2+}$  imbalance involves cleavage of cadherins and disassembly of CAJs (Herren *et al.*, 1998; Ito *et al.*, 1999; Vallorosi *et al.*, 2000; Steinhilber *et al.*, 2001). Furthermore, in the case of metastasis of epithelia tumors, inhibition of E-cadherin-mediated cell-cell adhesion is promoted by cleavage and secretion of extracytoplasmic E-cadherin by MMPs (Lochter *et al.*, 1997; Christofori and Semb, 1999; Noe *et al.*, 2001). Although in some cell cultures PS1 was found mainly in the ER-Golgi system (De Strooper *et al.*, 1997; Cupers *et al.*, 2001), in cell cultures with extensive cell-cell contacts and in tissues PS1 concentrates mainly at the plasma membrane (Georgakopoulos *et al.*, 1999; Nowotny *et al.*, 2000; Xia *et al.*, 2001) and, in the

brain, this protein concentrates at synaptic contacts (Georgakopoulos *et al.*, 1999; Ribaut-Barassin *et al.*, 2000). In addition, PS1 has been detected in vesicles (Efthimiopoulos *et al.*, 1998) and at the surface of both *Drosophila* cells (Nowotny *et al.*, 2000) and adhesive lamellipodia (Schwarzman *et al.*, 1999). The plasma membrane localization of PS1 is entirely consistent with its role in the processing of cell surface transmembrane proteins including APP, Notch1 receptor and E-cadherin. Our data define a novel mechanism of CAJ disassembly (Figure 7C). Key steps in this mechanism are the proteolytic cleavage of E-cadherin by a PS1/ $\gamma$ -secretase activity at the membrane-cytosol interface and a novel MMP cleavage of extracellular E-cadherin that occurs close to the plasma membrane. E-Cad/CTF1, the product of the MMP cleavage, remains bound to catenins and anchored to the cytoskeleton (Figure 7C). The PS1/ $\gamma$ -secretase cleavage dissociates both E-Cad/CTF1 and full-length E-cadherin from the cytoskeleton and promotes solubilization of cytoskeletal  $\beta$ - and  $\alpha$ -catenin, thus promoting disassembly of the CAJs. That the PS1/ $\gamma$ -secretase E-cadherin cleavage is significantly increased upon cell apoptosis or  $\text{Ca}^{2+}$  imbalance suggests the existence of signaling mechanisms that regulate the activity of the PS1/ $\gamma$ -secretase proteolytic system.

It was shown recently that under conditions that favor cell-cell adhesion, PS1 stabilizes the E-cadherin-catenin adhesion complex (Baki *et al.*, 2001). Combined with the present data, this suggests that with regard to the formation and disassembly of CAJs, PS1 may have two distinct activities. (i) Under conditions promoting cell-cell adhesion, incorporation of PS1 into the E-cadherin-catenin complex results in the stabilization of cell-cell adhesion (Baki *et al.*, 2001). (ii) In contrast, under conditions of cell-cell dissociation or apoptosis, PS1 promotes disassembly of adherens junctions, thus facilitating cell separation. Having both activities in the same polypeptide may represent an efficient and quick way for the formation and dissolution of cell-cell contacts. Interestingly p120<sup>cas</sup>, a protein that like PS1 binds the cytoplasmic juxtamembrane region of E-cadherin, has also been shown to have either positive or negative effects on CAJ-mediated cell-cell adhesion (Thoreson *et al.*, 2000).

Induction of the PS1/ $\gamma$ -secretase cleavage of E-cadherin results in the release of the E-Cad/CTF2- $\beta$ -catenin complex from the cytoskeleton and in a significant increase in the soluble cytosolic  $\beta$ -catenin (Figure 5C). Free cytosolic  $\beta$ -catenin is a key potent regulator of the Wnt signaling pathway. It translocates to the nucleus where it complexes with Lef-1/Tcf transcription factors to activate expression of target genes (Polakis, 2000). Thus, production of the E-Cad/CTF2- $\beta$ -catenin complex, rather than free  $\beta$ -catenin, may provide a large pool of complexed inactive  $\beta$ -catenin from where it could be released according to the transcriptional needs of the cell. The potential importance of E-Cad/CTF2 in signal transduction is indicated in recent studies using artificial constructs overexpressing soluble cytoplasmic cadherin sequences. These constructs bind  $\beta$ -catenin and inhibit the  $\beta$ -catenin/Lef-1/Tcf signaling events (Sadot *et al.*, 1998; Gottardi *et al.*, 2001). In addition, by sequestering  $\beta$ -catenin, these constructs act as dominant-negative agents in cell growth and development (Sanson *et al.*,

1996; Gottardi *et al.*, 2001). Thus the PS1/ $\gamma$ -secretase-derived E-Cad/CTF2 may provide a cellular regulatory link between changes in the E-cadherin-based cell–cell adhesion and the Wnt signal transduction pathway. That PS1 $^{-/-}$  mouse embryos accumulate E-Cad/CTF1 indicates that PS1/ $\gamma$ -secretase processing of E-cadherins occurs early in development and may control cadherin-mediated developmental processes and cell fate decisions.

The PS1/ $\gamma$ -secretase cleavage of E-cadherin shows several important differences from the corresponding cleavages in APP and Notch. Recent data (Gu *et al.*, 2001; Sastre *et al.*, 2001; Yu *et al.*, 2001) suggest that the APP cleavage may not occur in the middle of the transmembrane sequence of APP that defines the C-terminus of A $\beta$  peptides, but rather closer to the cytoplasm about three residues inside the membrane. Our data show that the  $\gamma$ -secretase cleavage of E-cadherin takes place at the end of the transmembrane sequence probably outside the lipid bilayer at the membrane–cytoplasm interface, suggesting that an intramembranous cleavage may not be a requirement for the PS1/ $\gamma$ -secretase proteolytic system. Similarly to APP and Notch,  $\gamma$ -secretase cleaves a membrane-bound C-terminal fragment of E-cadherin resulting from an ectodomain cleavage. In contrast to full-length APP and Notch, however, which do not seem to be cleaved by  $\gamma$ -secretase, full-length E-cadherin may also be processed by this activity. Finally, our data suggest that E-cadherin mutants unable to bind PS1 are not cleaved by the PS1/ $\gamma$ -secretase system. It would be important to determine whether PS1 binding to APP or Notch is a requirement for the  $\gamma$ -secretase cleavage of these proteins.

Our findings have important implications for Alzheimer's disease because they raise the possibility that the PS1 FAD mutants affect the PS1/ $\gamma$ -secretase cleavage of several CAJ systems. We observed that in addition to E-cadherin, two more classic cadherins, N- and VE-cadherins, both of which display high homology to E-cadherin, are metabolized by the PS1/ $\gamma$ -secretase system (unpublished observations). Both E- and N-cadherins have been found at the synapse and are reported to play key roles in synaptic structure and function (Fannon and Colman, 1996; Tang *et al.*, 1998; Tanaka *et al.*, 2000). In addition, VE-cadherin plays important roles in endothelial permeability (Sandoval *et al.*, 2001), vascular integrity (Corada *et al.*, 1999) and survival signaling of endothelial tissue (Carmeliet *et al.*, 1999). It will be important to examine what roles, if any, the PS1/ $\gamma$ -secretase cleavage plays in the functions of the above cadherin systems. Furthermore, it will be important to determine any potential effects of the PS1 FAD mutants on the  $\gamma$ -secretase processing of classic cadherins and their functions. However, our data suggest that potential therapeutic treatments targeting  $\gamma$ -secretase may also affect the proteolytic processing and/or function of a number of cadherin adhesion/signaling systems.

## Materials and methods

### Materials and antibodies

GM6001 was obtained from Chemicon International, Inc., STS was from Sigma, and ionomycin and Z-DEVD-FMK were from Calbiochem. L-685,458 and its inactive analogue were kindly provided by Dr M.S.Shearman (Merck Research Labs). Rabbit polyclonal antibody

R222 was raised against amino acids 2–12 of human PS1/NTF, and mouse monoclonal antibody 33B10 is specific for residues 331–350 of human PS1/CTF (Georgakopoulos *et al.*, 1999). Rabbit polyclonal antibody R1 was raised against human APP<sub>751</sub> amino acids 729–751 (Anderson *et al.*, 1989). Anti-E-cadherin (clone C36), anti- $\beta$ -catenin and anti- $\alpha$ -catenin monoclonal antibodies were obtained from BD Transduction Laboratories; antibody H108 against E-cadherin ectodomain was obtained from Santa Cruz Biotechnology, Inc.

### Mouse embryo preparation

Wild-type and PS1 knock-out mouse embryos (Baki *et al.*, 2001) were collected at day 18.5 post-coitum and solubilized by mechanical dissociation and sonication in RIPA buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 $\times$  Complete protease inhibitor cocktail, Roche). A 50  $\mu$ g aliquot of extract was analyzed by western blot.

### Cell cultures and transfections

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum, penicillin and streptomycin in 5% CO<sub>2</sub> at 37°C. Fibroblast cell lines derived from either wild-type (PS1 $^{+/+}$ ) or PS1 knock-out (PS1 $^{-/-}$ ) mice were stably transfected with human E-cadherin as described (Baki *et al.*, 2001). A431 cells were from the American Type Culture Collection. Stable transfectants of PS1 cDNA in HEK293 cells were prepared as described (Georgakopoulos *et al.*, 1999). A431D cells stably transfected with wild-type or GGG759-761AAA mutant E-cadherin were provided by Dr A.B.Reynolds.

### Subcellular fractionation

Confluent A431 cells (one 100 mm dish) were rinsed and scraped with 4°C phosphate-buffered saline (PBS). Cells were then placed into 1 ml of buffer A (20 mM Tris–HCl pH 7.5, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 $\times$  Complete protease inhibitor cocktail, Roche), passed through a 27 gauge needle 10 times, and the obtained cell lysate was centrifuged at 500 g for 10 min at 4°C. The supernatant was then centrifuged at 120 000 g for 45 min at 4°C to separate the cytosolic and crude membrane fractions. The pellet (crude membrane fraction) was washed twice with buffer A and resuspended by sonication in 400  $\mu$ l of buffer A containing 1% Triton X-100. The suspension was incubated at 4°C for 30 min and then centrifuged at 120 000 g for 45 min at 4°C to separate the membrane and Triton X-100-insoluble fractions. The pellet (Triton X-100-insoluble fraction) was washed twice with buffer A and solubilized by sonication in RIPA buffer. A 20  $\mu$ g aliquot of proteins from the different fractions was analyzed by SDS–PAGE.

### Immunoprecipitations (IPs), immunoblotting, immunofluorescence and confocal microscopy

For western blot analysis of cell extracts, cells were washed with PBS and then solubilized in RIPA buffer. For IPs, cells were solubilized in HEPES buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 1 $\times$  Complete protease inhibitor cocktail, Roche) containing 1% digitonin (IPs with I-R222 and PI-R222 antibodies) or 1% Triton X-100 (IPs with  $\beta$ -catenin and desmoglein antibodies). Following centrifugation at 17 000 g for 10 min, supernatants (1 mg of protein) were pre-cleared with protein A– or protein G–agarose (Pierce) for 2 h. Supernatants were then incubated with antibodies overnight at 4°C and treated for 2 h with protein A– agarose (polyclonal antibodies) or with protein G–agarose (monoclonal antibodies). IPs were washed with HEPES buffer containing either 1% digitonin or 1% Triton X-100 and analyzed by SDS–PAGE. Immunofluorescence and confocal microscopy were performed as described (Georgakopoulos *et al.*, 1999). Briefly, cells were plated on 22  $\times$  22 mm collagen-coated glass coverslips and fixed in cold methanol for 10 min at  $-20^{\circ}$ C. Following washing in TBS (25 mM Tris–HCl pH 7.4, 150 mM NaCl), cells were treated with 10% goat serum in SuperBlock (Pierce) for 1 h, incubated overnight with primary antibody (1:100), washed in TBS and then incubated with species-specific Alexa Fluor™ secondary antibody conjugates (Molecular Probes). Cells were washed with TBS, mounted with Prolong antifade kit (Molecular Probes) and photographed on a Leica confocal laser scanning microscope.

### Purification, mass spectrometry and N-terminal sequence analysis of E-cadherin C-terminal fragments

A431 cells treated with 1  $\mu$ M STS for 6 h were solubilized by sonication in RIPA buffer. A 50 mg aliquot of protein extract was pre-cleared with 20  $\mu$ g of H108 antibody, 20  $\mu$ g of unrelated monoclonal antibody and a mixture of protein A– and protein G–agarose (Pierce). The pre-cleared supernatant was treated overnight with 120  $\mu$ g of C36 anti-E-cadherin

antibody, and immunoglobulins were precipitated with 500  $\mu$ l of protein G-agarose. Immunoprecipitates were split into two samples and submitted to SDS-PAGE. One sample was stained with GelCode blue stain reagent (Pierce) and the 33 and 38 kDa fragments of E-cadherin were submitted to MALDI-MS Peptide Mass Mapping after in-gel digestion (Dr M.A.Gawinowicz, HHMI/Columbia University Protein Core Facility). The second sample was transferred on to a polyvinylidene difluoride (PVDF) membrane and the 38 and 33 kDa bands corresponding to E-Cad/CTF1 and E-Cad/CTF2, respectively, were subjected to sequential derivitization and cleavage of N-terminal amino acids by Edman chemistry followed by reverse-phase high performance liquid chromatography, (automated Applied Biosystems Procise 492 Peptide Sequencer, New York University Protein Analysis Facility).

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