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Signal peptide peptidase dependent cleavage of type II transmembrane substrates releases intracellular and extracellular signals

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Abstract

The intramembrane-cleaving proteases (I-CLiPs) presenilin-1 and -2 (PS1 and PS2), signal peptide peptidase (SPP) and the Site-2 protease (S2P) catalyze critical steps in cell signaling and are implicated in diseases such as Alzheimer's disease, hepatitis C virus (HCV) infection and cholesterol homeostasis. Here we describe the development of a cellular assay based on cleavage of the transmembrane sequence of the HCV core protein precursor, releasing intra- and extra-cellular signals that represent sequential signal peptidase and SPP cleavage, respectively. We find that the SPP inhibitor (Z-LL)2-ketone (IC_{50}=1.33 μM) and the γ-secretase potent inhibitors NVP-AHW700-NX (IC_{50}=51 nM) and LY411575 (IC_{50}=61 nM) but not DAPT dose dependently inhibited SPP but not signal peptidase cleavage. Our data confirm that type II orientated substrates, like the HCV transmembrane sequence, are sequentially cleaved by signal peptidase then SPP. This dual assay provides a powerful tool to pharmacologically analyze sequential cleavage events of signal peptidase and SPP and their regulation.

Keywords: I-CLiP (intramembrane cleaving protease); γ-Secretase inhibitor; SPP (single peptide peptidase) splice variant; S2P (Site-2 protease) cleavage; Reporter gene assay

1. Introduction

The family of intramembrane-cleaving proteases (I-CLiPs) include the presenilins (PS1 and PS2) and the signal peptide peptidase (SPP)-type aspartic proteases, in addition to the Site-2 protease (S2P)-type metalloproteases and the Rhomboid-type serine proteases (Ponting et al., 2002; Weihofen et al., 2002; Weihofen and Martoglio, 2003). I-CLiPs catalyze peptide bond hydrolysis within the plane of cellular membranes to liberate transcription factors and signaling molecules (Weihofen and Martoglio, 2003). The aspartic I-CliPs, presenilins and SPP, contain motifs of a GxGD aspartic protease, namely YD and LGLGD motifs. Both motifs are located in adjacent and opposing transmembrane domains, providing a molecular scaffold that is consistent with cleavage of membrane-spanning peptides (Weihofen et al., 2002; Steiner et al., 2000; Wolfe et al., 1999). These proteases also contain a highly conserved PAL motif that appears to be critical for conferring proteolytic activity (Tomita et al., 2001). S2P-type metalloproteases contain the consensus motifs HExxH and LDG within the active site and is also located in the transmembrane domain (Weihofen and Martoglio, 2003; Rawson, 2003). The Rhomboid-1 serine protease and its family contain a conserved sequence GxSG in addition to conserved asparagine and histidine residues that together create a catalytic site within the membrane plane (Weihofen and Martoglio, 2003; Urban et al., 2001; Urban and Wolfe, 2005).

SPP is a multi-transmembrane protein found in animals and plants and promotes intramembrane proteolysis of distinct signal peptides after they have been cleaved from newly synthesized secretory or membrane proteins (Ponting et al., 2002; Weihofen et al., 2002; Weihofen and Martoglio, 2003; Lemberg and Martoglio, 2002; Moliaka et al., 2004; Urny et al., 2003; Nyborg et al., 2004). SPP is essential for the generation of signal peptide-derived human lymphocyte antigen (HLA)-E-binding epitopes, which play a crucial role in immune

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surveillance (Lemberg et al., 2001). SPP is also involved in the maturation of hepatitis C virus (HCV) core protein and thought to be essential for the hepatitis C virus (HCV) life cycle (McLauchlan et al., 2002). Furthermore, SPP plays a role in cleaving preprolactin to release a signal peptide fragment that binds Ca^{2+}/calmodulin (Martoglio and Dobberstein, 1998; Lemberg et al., 2001; Lemberg and Martoglio, 2002). Four homologous SPP-like proteins (SPPL) have been identified in addition to SPP (Ponting et al., 2002; Weihofen et al., 2002).

PS1 is the catalytic subunit of the hetero-oligomeric γ-secretase complex composed PS1, Nicastrin, Pen2 and Aph1 (Haass and Steiner, 2002). We have previously shown that potent inhibitors of γ-secretase can block SPP using biochemical approaches (Weihofen et al., 2003). More recently, a study using an SPP reporter assay has also shown that inhibitors of γ-secretase can inhibit the function of SPP (Nyborg et al., 2004). This assay is based on the SPP-mediated cleavage of a transcription factor, namely ATF6, into the cytosol which activates luciferase (Nyborg et al., 2004). To further understand the sequential cleavage of a type II transmembrane substrate, we have developed an improved dual reporter assay that can sequentially measure signal peptidase then SPP cleavage. We fused reporter moieties on both NH2 and COOH terminals of the HCV transmembrane sequence, which is contiguous to the viral structural proteins core and E1. The yeast DNA binding protein Gal4 and transcriptional activator VP16 or a β1 epitope was introduced into the NH2-terminus, whereas a secreted enhanced alkaline phosphatase (SEAP) was fused to the COOH-terminus. Here we show the release of a reporter signal (secreted enhanced alkaline phosphatase, SEAP) into the culture medium after signal peptidase cleavage in addition to the SPP-mediated liberation of a transcription factor (Gal4/VP16) into the cytosol. Using this approach we gain insights into the topology of a type II transmembrane substrate and its colocalisation with SPP in the endoplasmatic reticulum compartments. Importantly, we are now able to confirm that signal peptidase and SPP cleavages are sequential in nature.

2. Materials and methods

2.1. Molecular biology and description of constructs

For the signal peptidase/SPP cleavage assay, the coding sequence of the Gal4 DNA binding domain (GenBank #AAA34626) was fused to the coding sequence of the VP16 transactivation domain (Baron et al., 1997; Seipel et al., 1992) with a five residue linker LPLQL between Gal4 and VP16 to create a Gal4/VP16 fusion. This was cloned upstream of the HCV coding sequence (GNLPGCSFSIFLLA//LLSCLTVPASA/YQVR) where the signal peptidase (/) and SPP (//) sites are indicated (Hope and McLauchlan, 2000). Downstream of the HCV, the coding sequence of secreted enhanced alkaline phosphatase (SEAP; GenBank #AAB64402) without the signal peptide residues 1–17 was cloned. This created the [Gal4/VP16]-HCV-[SEAP] substrate construct. The Gal4/VP16 was replaced with the β1 epitope tag (β1, MDAEFRHD) and the HCV sequence extended by an extra upstream 13 residues (GVRVLEDGVNYAT). This created the [β1]-HCV-[SEAP] substrate construct. The signal peptidase and SPP cleavage sites were mutated by Quick Change PCR (Promega). Cloning was done using standard PCR and restriction digest procedures (see Fig. 1A for further details). The above constructs were inserted into the pCI mammalian expression vector (Promega). Integrity of constructs was verified by DNA sequencing. Similar methods were used to generate wild type, signal peptidase and SPP mutant prolactin substrate constructs [Gal4/VP16]-Prl-[SEAP] (see Fig. 2C for comparison with [Gal4/VP16]-HCV-[SEAP] constructs). The plasmids for the sterol regulatory element binding protein (SREBP) assay, including, sterol regulatory element (SRE)-luciferase, CMV-Renilla, SREBP-cleavage activating protein (SCAP) and Insig-1 are previously described (Hua et al., 1996; Yang et al., 2002).

2.2. Cell culture

For luciferase and SEAP assays, cells were plated in 96-well tissue culture grade plates as a suspension of $1–2 \times 10^4$ cells per well in 100 μl of Nutrient mixture F12 (HAM) (Invitrogen) supplemented with 10% fetal calf serum for CHO cells or in DMEM low glucose, 40 mg/ml L-proline (Invitrogen) supplemented with 10% fetal calf serum for HEK293 cells. Culture medium was supplemented with 1% penicillin/streptomycin (Invitrogen). Cells, grown to 80% confluence, were transfected using Lipofectamine (Invitrogen) or FuGene6 (Roche) according to manufacturer instructions. For the SREBP cleavage assay, CHO cells were grown Nutrient F12 (HAM) medium containing 10% lipoprotein deficient serum (Sigma) supplemented with 50 μM Mevalonate (Sigma) and 50 μM Mevastatin (Sigma). 25-OH cholesterol (Sigma) was added at a concentration of 1 μg/ml.

2.3. Luciferase, SEAP and cell viability assays

To quantify the luciferase levels, at 24 or 48 h post-transfection, cells were solubilized with solubilization buffer (Promega), Dual-Glow luciferase substrate (Promega) was added and light emission measured in a luminometer. The firefly luciferase levels were normalized to *Renilla* luciferase levels. To quantify the level of secreted SEAP, supernatants were mixed with dilution buffer (Roche), heated for 30 min at 60 °C, mixed with inactivation buffer and incubated 5 min at room temperature. Subsequently, substrate reagents were added and gently rocked for 10 min at room temperature, light emission was quantified in a luminometer. The viability assays, (3 (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and lactate dehydrogenase (LDH) (Promega), were performed following manufacturer instructions.

2.4. Western blotting

Cell pellets were resuspended in PTxE buffer (Phosphate buffered saline (PBS) supplemented with 1% Triton X-100 and

100 μM EDTA) and boiled in Laemmli buffer containing 2% β-Mercaptoethanol. Samples were electrophoresed on Tris-glycine 4–15% gradient gel (Invitrogen) and the proteins transferred onto a nitrocellulose membrane. Membranes were blocked in 5% milk, 1% BSA in TBST buffer (Tris buffered saline (TBS) supplemented with 0.05% Tween-20) then incubated with appropriate primary antibodies. After washing in TBST buffer secondary antibodies were applied. Enhanced chemiluminescence (Super Signal Femto, Pierce) was visualized by exposure to Hyperfilm ECL (Amersham Biosciences).

2.5. Immunostaining studies

COS7 cells were plated on glass-slides coated with poly-D-lysine (Becton Dickinson) at a density of approximately 5×10^4 cells/ml in growing medium (DMEM low glucose, 20 mg/l L-proline, L-glutamine and 10% fetal calf serum). Cells were transfected using FuGene6 as described by the manufacturer (Roche). Two days later, cells were fixed for 10 min with 4% paraformaldehyde and 11% sucrose in PBS, neutralized with two washes of 0.1 M glycine in PBS and rinsed three times with PBS. Cells were then solubilized for 1 h in blocking solution (0.4% saponin, 1% BSA and 2% heat inactivated horse serum in PBS) and incubated with appropriate primary antibodies. After washing secondary antibodies were applied for 1 h at room temperature. Excess secondary antibodies were removed by 3 sequential washes of 0.4% saponin in PBS. Slides were ethanol dried and mounted with Prolong (Molecular Probes). Samples were visualized on a Leica DMR confocal microscope.

2.6. Antibodies and staining reagents

Primary antibodies used include, β1 monoclonal antibody (mAb) (Dr M. Zurini, Novartis) (Schradrer-Fisher and Paganetti, 1996), Gal4 mAb (Santa Cruz Biotech), rat anti-HA (Roche) or rabbit anti-calnexin N-terminus (Santa Cruz Biotech). Secondary antibodies used for Western blotting were anti-mouse HRP conjugated and diluted according to manufacturer instructions (Sigma). Secondary antibodies used for immunostaining were as follows, anti-mouse Cy3 (Jackson Lab), anti-rat Cy3 (Jackson Lab) or anti-rabbit Cy2 (Jackson Lab). ToPro3 (Molecular Probes) was utilized to stain nuclei.

2.7. Compounds

The γ-secretase inhibitors DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester; WO 9822494), (Z-LL)2-ketone, NVP-AHW700-NX ({(1*S*,2*R*,4*R*)-1-Benzyl-4-[(*S*)-1-(7-chloro-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[*e*][1,4]diazepin-3-ylcarbamoyl)-ethylcarbamoyl]-2-hydroxy-5-phenyl-pentyl}-carbamic acid *tert*-butyl ester) and LY411575 ((*S*)-2-[2-(3,5-Difluorophenyl)-acetylamino]-*N*-((*S*)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[*b*,*d*]azepin-7-yl)-propionamide; WO 9828268; Friedmann et al., 2004), and the signal peptidase inhibitor MeOSuc-Ala-Ala-Pro-Val-Chloromethylketone (Bachem N-1055) were pre-diluted in serum-free medium and added as 10-fold concentrate solution to the cells 1 h after transfection. To deregulate cholesterol transport, CHO cells were treated for 15 h with 1 μM of the 2,3-oxidosqualenelanosterol cyclase inhibitor, called 3β-(2-Diethylaminoethoxy)androst-5-en-17-one, HCl (U18666A) (Calbiochem) (Liscum and Faust, 1989).

3. Results

3.1. Proteasomal degradation and endoplasmic reticulum localization of the HCV substrates with SPP (Fig. 1)

We first determined the expression levels of wild-type-, ΔSPP- and ΔSP-HCV fusion substrates using transiently transfected HEK293 cells. Similar results were obtained with both types of the HCV fusion substrates, [β1]-HCV-[SEAP] and [Gal4/VP16]-HCV-[SEAP] (Fig. 1B). Expression of ΔSP-HCV fusion substrates but not wild-type or ΔSPP was detected by Western blotting. Rapid proteasomal clearance after signal peptidase cleavage could result in low levels of wild-type- and ΔSPP-HCV fusion substrates. To test this hypothesis, we determined the expression levels of wild-type-HCV fusion substrates in the presence of the proteasomal inhibitor Lactacystin (5 μM added 24 h post transfection). Inhibition of the proteasome by Lactacystin resulted in the detection of both wild-type versions of [β1]-HCV-[SEAP] and [Gal4/VP16]-HCV-[SEAP]. Taken together these results show that signal peptidase cleaved HCV fusion substrates are more rapidly cleared by proteasomal degradation than signal peptidase mutated HCV fusion substrates. Mutation of the signal peptidase site or blockade of the proteasome stabilizes the full-length HCV substrate.

In order to study the subcellular localization of the HCV proteins and verify overlap of HCV substrates with its cleaving enzyme SPP, the Gal4/VP16 domain of the [Gal4/VP16]-HCV-[SEAP] construct was replaced with the β1 epitope tag (MDAEFRHD) (Fig. 1A). Similar to the [Gal4/VP16]-HCV-[SEAP], SEAP should be liberated by signal peptidase cleavage from [β1]-HCV-[SEAP] once it is translocated into the endoplasmic reticulum lumen and recovered in the conditioned medium. Like Gal4/VP16, the β1 epitope faces the cytosol and subsequent processing by SPP should liberate the N-terminal β1 epitope into the cytosol. The β1 epitope was not recovered from cell lysates presumably due to rapid clearance of this tag coupled with a technical difficultly in the purification and detection of this short peptide from the cell lysate. The [β1]-HCV-[SEAP] substrate was located in the endoplasmic reticulum and highly colocalised with the endoplasmic reticulum marker calnexin (Fig. 1C). The [β1]-HCV-[SEAP] substrate was also colocalised with transiently transfected HA-SPP supporting the idea that transiently transfected [Gal4/VP16]-HCV-[SEAP] and [β1]-HCV-[SEAP] substrates are likely to be processed by SPP within the endoplasmic reticulum compartments (Fig. 1C).

3.2. Subsequent cleavage by signal peptidase and SPP can be measured by SEAP and luciferase respectively (Fig. 2)

As expected, we found that the wild-type [Gal4/VP16]-HCV-[SEAP] is cleaved by signal peptidase giving SEAP activity

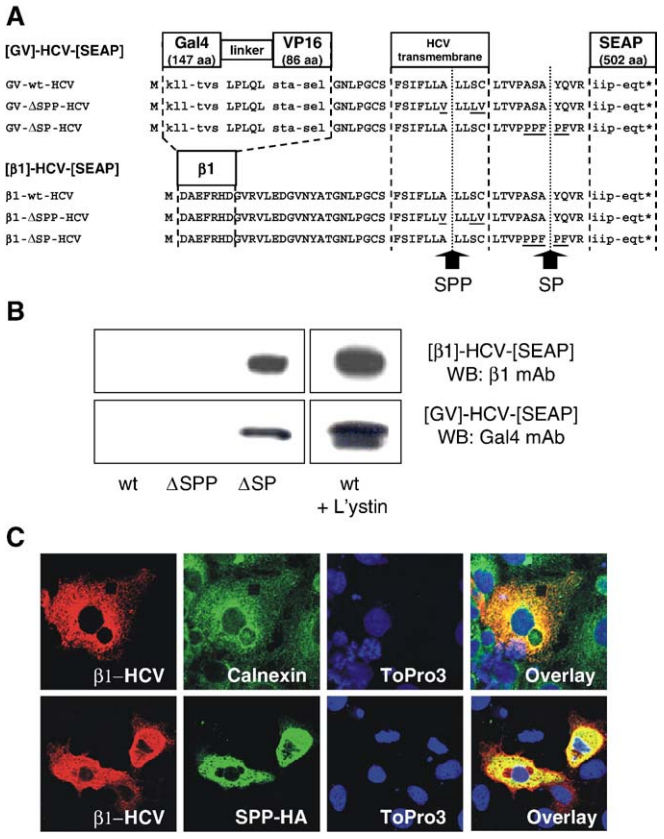

Fig. 1. Details of constructs, their expression levels and subcellular localization with SPP. (A) The [Gal4/VP16]-HCV-[SEAP] is composed nucleotides 503–588 of HCV core coding region (Hope and McLauchlan, 2000) fused downstream of Gal4/VP16 transcription factor, while the [β1]-HCV-[SEAP] is composed nucleotides 463–588 of HCV core coding region fused downstream to the β1 epitope tag. All constructs have secreted enhanced alkaline phosphatase (SEAP) fused downstream of the HCV sequence. Wild type (wt), mutant SPP (ΔSPP) and mutant signal peptidase (ΔSP) constructs are shown as well as the 3 first and 3 last amino acids of Gal4, VP16 and SEAP. (B) Shown are the Western blots (WB) of cell pellet lysates of transiently transfected HEK293 cells with wild type, ΔSPP and ΔSP constructs of [β1]-HCV-[SEAP] using β1 mAb (1 μg/ml) as primary antibody and [Gal4/VP16]-HCV-[SEAP] using Gal4 mAb (1:100) as primary antibody. Cells were treated with or without the proteasomal inhibitor Lactacystin (5 μM added 24 h post transfection). 80 μg total protein loaded per lane. (C) COS7 cells were transiently transfected with [β1]-HCV-[SEAP] wild type construct, without or with SPP-HA. Cells were permeabilised with 0.4% saponin and stained with β1 mAb (HCV construct marker), HA antibody (SPP-HA), calnexin N-terminal (endoplasmic reticulum marker) and/or ToPro3 (nuclear marker). Staining was viewed by Confocal, 488 nm laser. Primary antibodies used include, mouse β1 (1 μg/μl stock diluted to 1:900) (Schradrer-Fisher and Paganetti, 1996); rat anti-HA (diluted 1:300, Roche); or rabbit anti-calnexin N-terminus (diluted 1:200, Santa Cruz Biotech). Secondary antibodies were as follows, anti-mouse IgG Cy3 (diluted 1:400, Jackson Lab) or anti-rabbit Cy2 (diluted 1:100, Jackson Lab). ToPro3 (Molecular Probes) was diluted to 0.5 μM in PBS and utilized to stain nuclei.

(Fig. 2A). Predictably, mutation at SPP (ΔSPP) had no effect on signal peptidase cleavage-dependent SEAP activity, whereas mutation at signal peptidase (ΔSP) prevented signal peptidase cleavage and inhibited release of SEAP (Fig. 2A). The wild-type [Gal4/VP16]-HCV-[SEAP] substrate is also cleaved by SPP as measured by luciferase activity. This SPP-dependent cleavage is reduced by the γ-secretase inhibitor, LY411575, in agreement with our previous report (Weihofen et al., 2003). Mutation at SPP (ΔSPP) rendered the ΔSPP HCV substrate insensitive to γ-secretase inhibition but had little effect on control levels of luciferase activity (Fig. 2A). The [β1]-HCV-[SEAP] construct lacks Gal4/VP16 moiety and did not induce luciferase activity (data not shown). To further confirm that the release of SEAP reflects signal peptidase processing and that luciferase activity reflects SPP cleavage, we generated [Gal4/VP16]-Prl-[SEAP] constructs (Fig. 2C). We found that the wild-type [Gal4/VP16]-Prl-[SEAP] is cleaved by signal peptidase and SPP to give SEAP and luciferase activities, respectively (Fig. 2B). Mutation in signal peptidase (ΔSP) prevented signal peptidase cleavage and thus also downstream SPP cleavage, inhibiting both SEAP and luciferase activities (Fig. 2B). Mutation in SPP (ΔSPP) had no effect on SEAP cleavage, as expected and showed a significant reduction in luciferase activity compared to the wild type Prl substrate (Fig. 2B).

3.3. An inhibitor of signal peptidase reduces SEAP activity (Fig. 3)

To confirm that signal peptidase and SPP reporter assays are regulated by pharmacological tools and to support the ΔSPP and ΔSP mutational data, we determined the effects of a signal peptidase inhibitor on SEAP cleavage and luciferase activity using the wild-type [Gal4/VP16]-HCV-[SEAP] construct. Similar to mutation in the signal peptidase cleavage site (ΔSP) (Fig. 1B), the weak but irreversible signal peptidase inhibitor MeOSuc-Ala-Ala-Pro-Val-Chloromethyl Ketone

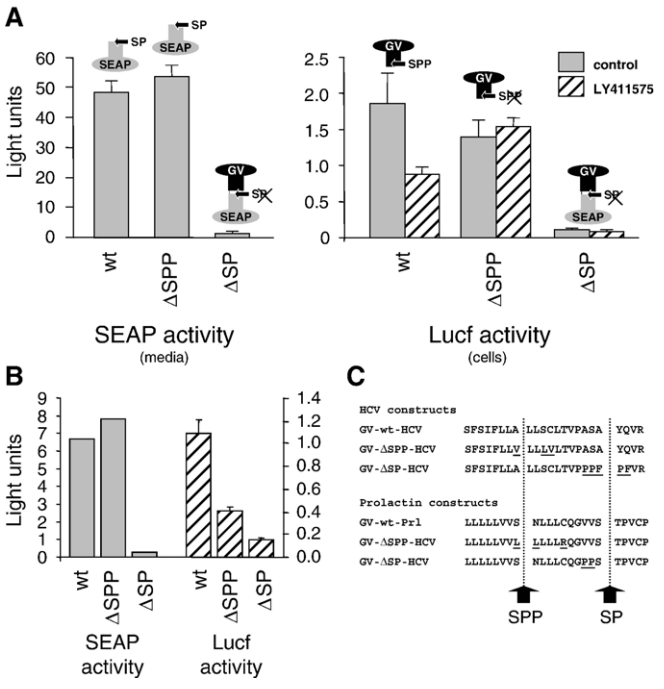

Fig. 2. Signal peptidase and SPP cleavage assays. CHO cells were transiently transfected with wt, ΔSP and ΔSPP constructs of (A) [Gal4/VP16]-HCV-[SEAP] and (B) [Gal4/VP16]-Prl-[SEAP]. Forty-eight hours later the conditioned medium was removed and assayed for SEAP activity. Cell monolayer was solubilized and the relative levels of induced luciferase activity were quantified. (C) The [Gal4/VP16]-HCV-[SEAP] and [Gal4/VP16]-Prl-[SEAP] transmembrane regions with signal peptidase and SPP cleavage sites is shown.

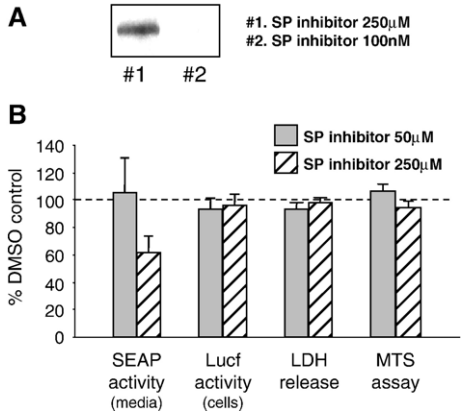

Fig. 3. Regulation of SEAP cleavage by a signal peptidase inhibitor. CHO cells were transiently transfected with wild type [Gal4/VP16]-HCV-[SEAP] construct and treated with the signal peptidase inhibitor MeOSuc-Ala-Ala-Pro-Val-Chloromethylketone. Forty-eight hours later all assays were performed. (A) Western blotting using Gal4 mAb (1:100), 80 μg total protein loaded per lane. (B) The conditioned medium was removed and assayed for SEAP activity. Cell monolayer was solubilized and the relative levels of induced luciferase activity were quantified. LDH release and MTS assays were performed to measure the effects of compounds on cellular viability. An increase in LDH and a decrease in MTS normally depict compound induced cell toxicity; no significant changes in viability were detected between 50 and 250 μM of the signal peptidase inhibitor.

(Bachem) (250 μM) prevented signal peptidase cleavage and allowed detection of full-length substrate by western blotting (Fig. 3A). As expected the signal peptidase inhibitor reduced SEAP activity. At a concentration of 250 μM approximately 40% inhibition of SEAP activity occurred with no changes in cell viability as determined by LDH and MTS assays (Fig. 3B). At higher concentrations this compound resulted in cellular toxicity and thus prevented complete dose-dependent inhibition of SEAP activity (data not shown). The 40% reduction of signal peptidase activity by this signal peptidase inhibitor may still allow for partial SPP cleavage. And since the Gal4/VP16 moiety is a strong transcription factor, small levels of Gal4/VP16 released by SPP cleavage may be sufficient to drive lucifease activity.

3.4. γ-Secretase and SPP inhibitors reduce luciferase activity (Fig. 4)

A SPP inhibitor and some γ-secretase inhibitors dose dependently inhibit SPP (luciferase activity) (Fig. 4A) but did not affect signal peptidase cleavage (secretion of SEAP) (Fig. 4B). The luciferase signal was dose-dependently inhibited by the γ-secretase inhibitors NVP-AHW700-NX (IC_{50}=51 nM) and LY411575 (IC_{50}=61 nM) but not by DAPT (Fig. 4A). The SPP inhibitor (ZLL) 2-ketone (IC_{50}=1.33 μM) also dose-dependently inhibited HCV cleavage (Fig. 4A). These results are in full agreement with our previous studies (Weihofen et al., 2003). As described above (Fig. 2), mutation in SPP (ΔSPP) site resulted in a luciferase signal that was not regulated by γ-secretase inhibitors (Fig. 4C). This mutated ΔSPP substrate mediated a luciferase activity was also not affected by the SPP inhibitor (Z-LL)2-ketone (Fig. 4C).

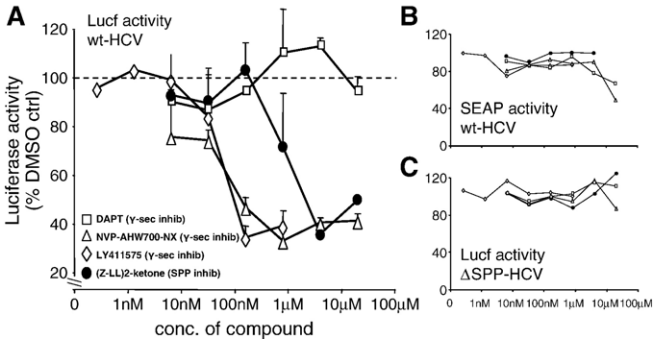

Fig. 4. Inhibition of luciferase activity by γ-secretase and SPP inhibitors. HEK293 cells were transiently transfected with wt (A, B) and δSPP (C) [Gal4/VP16]-HCV-[SEAP] constructs and treated with the γ-secretase inhibitors (DAPT, NVP-AHW700-NX, and LY411575) and the SPP inhibitor ((Z-LL)2-ketone). Forty-eight hours later the conditioned medium was removed and assayed for SEAP activity (B). Cell monolayer was solubilized and the relative levels of induced luciferase activity were quantified (A, C).

3.5. γ-Secretase inhibitors do not regulate S2P (Fig. 5)

The site-2 protease (S2P) cleaves the sterol regulatory element binding protein (SREBP) after a prior cleavage event by site-1 protease (S1P) (Rawson, 2003; Yang et al., 2002). These cleavage events result in the release of the transcription factor N-terminus of SREBP (N-SREBP). In this study, we have made use of a SREBP assay, that measures release of the SREBP-N which drives expression of a luciferase reporter gene containing SRE (Hua et al., 1996). We first validated this assay using a number of controls. U18666A is a cell-permeable, amphiphilic amino-steroid that alters intracellular membrane protein trafficking. It is thought to impair intracellular biosynthesis and transport of LDL-derived cholesterol, by inhibiting 2,3-oxidosqualene-lanosterol cyclase (Liscum and Faust, 1989). In this SREBP assay, U18666A increased luciferase levels most likely as a result of impaired cholesterol transport (Fig. 5A). Furthermore, we found that addition of a derivative of cholesterol (25-OH-cholesterol) caused luciferase levels to decrease. The overexpression of Insig-1 which is reported to cause endoplasmic reticulum retention of the

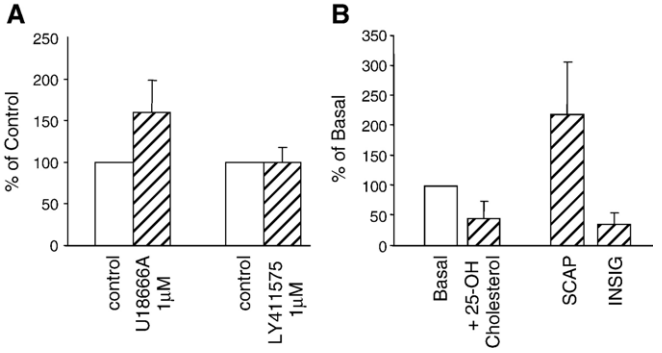

Fig. 5. γ-Secretase inhibitors do not prevent S2P cleavage. CHO cells were transiently transfected with pSRE-luciferase and pCMV-renilla with or without pSCAP or pInsig1. Cells were treated with 25-OH cholesterol, U18666A or LY411575 as indicated (A, B).

SREBP-SCAP complex also reduced luciferase levels. On the other hand, overexpression of SCAP is thought to promote SREBP transport to the Golgi and accordingly increased luciferase levels (Fig. 5B). Despite LY411575 being a potent inhibitor of γ-secretase activity and also inhibiting SPP activity, we find that LY411575 fails to regulate S2P-dependent SREBP cleavage (Fig. 5A).

4. Discussion

The intramembrane proteolysis of HCV core protein from the E1 and E2 envelope glycoproteins ([core protein]-[signal sequence]-[E1 envelope glycoprotein]-[E2 envelope glycoprotein]) occurs at the endoplasmic reticulum membrane (Lemberg and Martoglio, 2002; McLauchlan et al., 2002; Hope and McLauchlan, 2000). Signal peptidase cleaves first between signal sequence and E1 envelope glycoprotein at an exposed site close to the membrane liberating E1 envelope glycoprotein. The core protein is released from the membrane upon subsequent intramembrane cleavage within the signal sequence mediated by SPP. This latter step is considered critical for proper maturation of the HCV core protein. Since correct processing of this HCV substrate requires sequential intervention of signal peptidase and SPP, we created a functional assay for these two proteases by placing the hinge region between the core protein and E1 envelope glycoprotein (that is sequentially processed by signal peptidase then SPP) upstream to the SEAP reporter and downstream to β1 or Gal4/VP16. Mutations at signal peptidase (ΔSP) and SPP (ΔSPP) cleavage sites were introduced to prevent cleavage at the respective sites (Fig. 1A). Taken together, our data show that the [Gal4/VP16 or β1]-HCV-[SEAP] substrate is first cleaved by signal peptidase allowing SEAP to be released into the endoplasmic reticulum lumen from where it is secreted into the media by exocytosis. Accordingly, SEAP activity can be measured in the condition media. Subsequent cleavage by SPP then allows the release of Gal4/VP16 or β1 into the cytosol.

The Gal4/VP16 translocates to the nucleus where it activates a Gal4 dependent reporter plasmid encoding luciferase. Activity of this reporter enzyme can be quantified in the cell lysate (Fig. 6A). Therefore, SEAP activity is a measure of signal peptidase cleavage while luciferase activity a measure of SPP cleavage (Fig. 6A).

In this study, we show that substitution of the signal peptide of SEAP by the HCV sequence is sufficient to induce translocation and secretion of SEAP. We provide evidence that SEAP faces the endoplasmic reticulum lumen and the Gal4/VP16 moiety faces the cytosol since SEAP activity can be recovered from the media and the Gal4/VP16 moiety can activate luciferase in the cytosol. These results are in agreement with the proteolytic processing predicted for a type II intramembrane cleaved substrate. The triple mutation ($A^{180}S^{183}C^{184}$ to $V^{180}L^{183}V^{184}$) surrounding the SPP cleavage site (Gal4/VP16-ΔSPP-HCV) increases the á-helix "index" by introducing Val and Leu residues and should reduce processing by SPP (McLauchlan et al., 2002). The five base pair mutation ($ASAYQ^{189–193}$ to $PPFPF^{189–193}$) surrounding the site recognized by signal peptidase also causes structural changes that is expected to inhibit signal peptidase cleavage (Lemberg and Martoglio, 2002) (Fig. 1A). We showed that mutation at the signal peptidase site (ΔSP) prevented SEAP activity. In agreement with the subsequent processing of HCV by signal peptidase followed by SPP, we also found that mutation in the signal peptidase site reduced SPP cleavage as measured by luciferase activity. We report that mutation at the SPP site (ΔSPP) rendered the ΔSPP HCV substrate insensitive to γ-secretase processing. Although this ΔSPP substrate was rapidly processed by the proteasome (Fig 1B) and showed luciferase signals, the luciferase activity was not sensitive to the SPP inhibitor (Z-LL)2-ketone (Fig. 5C) supporting the idea that the ΔSPP mutated substrate is no longer cleaved by SPP. Since the ASC to VLC mutation in the SPP cleavage site is reported to reduce SPP-dependent cleavage (McLauchlan et al., 2002) and the LY411575 is a known SPP inhibitor (Friedmann et al., 2004; Weihofen et al., 2003), we conclude that the residual luciferase activity is no longer part of the signal peptidase-SPP dependent cleavage mechanism. To prove further this claim, similar constructs to HCV were generated using the transmembrane region of prolactin, which is a substrate of signal peptidase and SPP cleavage (Fig 2C) (Martoglio and Dobberstein, 1998; Lemberg et al., 2001; Lemberg and Martoglio, 2002). Wild-type and ΔSP constructs of Prl showed similar results as that of the HCV substrates (Fig 2B). Importantly, Prl ΔSPP mutant construct showed significantly reduced levels of luciferase activity indicating that mutations in this region prevent SPP cleavage (Fig 2B). Taken together, these data indicate that the SEAP activity reflects signal peptidase cleavage, whereas the luciferase activity reflects SPP cleavage.

S2P catalyses the cleavage of transcription factors such as SREBPs and activating transcription factor 6 (ATF6). SREBPs control lipid homeostasis within cells (Rawson, 2003), while ATF6 is activated as a result of unfolded protein response (Sakai et al., 1996). SREBPs reside in the endoplasmic reticulum and are translocated to the Golgi apparatus by SCAP in response to low sterol levels. When sterol levels are high, the SREBP-SCAP complex is retained in the endoplasmic reticulum by

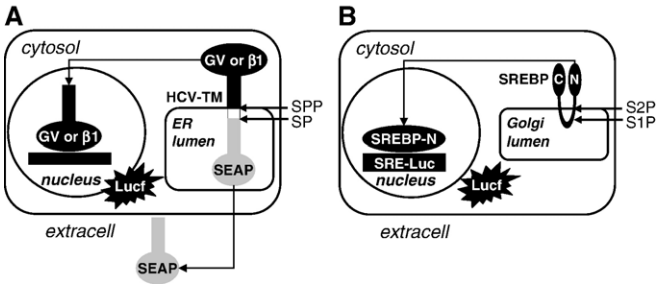

Fig. 6. Details of assays. (A) Cartoon of the signal peptidase/SPP assay. Cleavage of HCV substrates by signal peptidase releases SEAP as measured in conditioned media. Subsequent cleavage by SPP releases Gal4/VP16 into cytosol activating luciferase as measured in cell lysates. The [β1]-HCV-[SEAP] constructs lack Gal4/VP16 and do not induce luciferase activity (data not shown). (B) Schematic representation of the SREBP assay. Cleavage of SREBP in the Golgi by S1P and S2P results in the release of SREBP-N transcription factor that binds SRE elements driving luciferase expression.

Insig proteins (Rawson, 2003, Yang et al., 2002). Similar to γ-secretase and SPP, S2P cleaves SREBP within the plane of the Golgi membrane and can act only after a prior cleavage event by the Golgi localized S1P, which cleaves SREBP in its luminal part (Sakai et al., 1996). These cleavage events result in the release of N-SREBP, which contains transcriptional activation domains that up-regulate expression of genes containing SRE in their promoter (Rawson, 2003). Whilst the aspartic proteases PS1 and SPP are closely related, the S2P metalloprotease is thought to be a more distant family member. Recent reports have suggested that γ-secretase inhibitors may modulate γ-secretase activity by competing with the initial substrate binding sites that are distinct from the active site (Kornilova et al., 2005). Based on the fact that PS1, SPP and S2P all contain catalytic aspartate(s) within the plane of the membrane and to investigate the possibility that γ-secretase inhibitors are capable of inhibiting a more distant member of the I-CLiP family, we examined the effect LY411575 on S2P activity using a SREBP cleavage assay (Fig. 5A) (Hua et al., 1996). Similar to our SPP assay, the SREBP assay relies on the sequential cleavage of endogenous SREBP by S1P then S2P that releases the transcription factor SREBP-N which drives expression of a luciferase reporter gene containing SRE (Fig. 6B) (Hua et al., 1996). Under low sterol conditions, the luciferase levels are high as SREBP is transported to the Golgi by SCAP where it is cleaved to release SREBP-N. Our results show that γ-secretase inhibitors do not universally inhibit all I-CLiPs. Thus, although I-CLiPs have been grouped into a family of proteases based on transmembrane cleavage activity, we provide pharmacological evidences that suggest specific inhibitors can be developed against each of these family members.

During the development of our signal peptidase and SPP assay, we experimentally isolated two SPP isoforms by PCR. Total RNA was isolated from HEK293 cells using GlassMax kit (Invitrogen) and reverse-transcribed with Superscript II (Invitrogen). One clone differed at the N-terminus and was shorter by 33 residues (Δ61–93) as compared to SPP. Since this deletion occurred in the second transmembrane domain, it is likely to affect topology of the downstream transmembrane regions and inverse orientation of the catalytic domain. Such a splice variant may cleave type I substrates. The other clone differed at the central region, was shorter by 28 residues (Δ244–271) as compared to SPP-1 and lacked the GLGD motif where the second key aspartate is missing (data not shown). One might speculate that this splice variant could dimerise with SPP and exhibit a dominant-negative effect on wild-type SPP. Both SPP splice variants showed roughly 10% occurrence compared to SPP as determine by the number of clones found during PCR based cloning. Western blotting using the cell pellet lysates of transiently transfected HEK293 cells showed that molecular weights for SPP (377 residues, ~45 kDa) and for SPP-Δ244–271 (349 residues, ~40 kDa) were slightly larger than predicted, whereas the molecular weight for SPP-Δ61–93 (344 residues, ~40 kDa) was found as expected (data not shown). On a number of occasions, we also found that the transient overexpression of SPP in CHO cells showed high molecular band smears (data not shown) which may represent SPP dimers and/or large macromolecules (Friedmann et al., 2004). Immunocytochemistry of both splice variants transiently transfected CHO cells showed a similar subcellular localization as that of SPP; a perinuclear appearance possibly within the endoplasmic reticulum and Golgi apparatus (data not shown).

In conclusion, our signal peptidase and SPP reporter assays demonstrated the sequential cleavage of type II transmembrane substrates by signal peptidase then SPP. We have shown that a γ-secretase inhibitor is specific for PS1 and SPP inhibition, and does not inhibit S2P. Since PS1, SPP and S2P are major targets for therapeutic intervention, developing distinct and potent pharmacological inhibitors is pivotal to understanding the specific role of these I-CLiPs in the etiology of related diseases.

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