Characterization of the catalytic activity of the membrane-anchored metzalloproteinase ADAM15 in cell-based assays

Thorsten MARETZKY†*, Guangli YANG†, Ouathek OUERFELLI†, Christopher M. OVERALL‡§, Susanne WORPENBERG∥, Ulrich HASSIEPEN∥∥, Joerg EDER∥ and Carl P. BLOBEL*†

†Arthritis and Tissue Degeneration Program, Hospital for Special Surgery at Weill Medical College of Cornell University, 535 E 70th Street, New York, NY 10021, U.S.A., †Memorial Sloan-Kettering Cancer Center, New York, NY 10065, U.S.A., ‡Department of Oral Biological and Medical Sciences, University of British Columbia, Vancouver, BC, Canada V6T 1Z3, §Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3, and ∥Center for Protein Chemistry, Novartis Institutes for Biomedical Research, CH-4002 Basel, Switzerland

ADAM15 (a disintegrin and metalloproteinase 15) is a membrane-anchored metzalloproteinase, which is overexpressed in several human cancers and has been implicated in pathological neovascularization and prostate cancer metastasis. Yet, little is known about the catalytic properties of ADAM15. Here, we purified soluble recombinant ADAM15 to test for its ability to cleave a library of peptide substrates. However, we found no processing of any of the peptide substrates tested here, and therefore turned to cell-based assays to characterize the catalytic properties of ADAM15. Overexpression of full-length membrane-anchored ADAM15 or the catalytically inactive ADAM15E→A together with various membrane proteins resulted in increased release of the extracellular domain of the fibroblast growth factor receptor 2iiib (FGFR2iiib) by ADAM15, but not ADAM15E→A. This provided a robust assay for the characterization of the catalytic properties of ADAM15 in intact cells. We found that increased expression of ADAM15 resulted in increased FGFR2iiib shedding, but that ADAM15 was not stimulated by phorbol esters or calcium ionophores, two commonly used activators of ectodomain shedding. Moreover, ADAM15-dependent processing of FGFR2iiib was inhibited by the hydroxamate-based metzalloproteinase inhibitors marimastat, TAPI-2 and GM6001, and by 50 nM TIMP-3 (tissue inhibitor of metalloproteinases 3), but not by 100 nM TIMP-1, and only weakly by 100 nM TIMP-2. These results define key catalytic properties of ADAM15 in cells and its response to stimulators and inhibitors of ectodomain shedding. A cell-based assay for the catalytic activity of ADAM15 could aid in identifying compounds, which could be used to block the function of ADAM15 in pathological neovascularization and cancer.

Key words: ADAM (a disintegrin and metalloproteinase), ADAM15, fibroblast growth factor receptor 2 (FGFR2), ectodomain shedding.

INTRODUCTION

ADAMs (a disintegrin and metalloproteinase) are a family of cell-surface metzalloproteinases with key roles in signalling via the epidermal growth factor receptor, in the activation of Notch, in neurogenesis and angiogenesis, and in the shedding of various membrane bound proteins, including cytokines, growth factors and adhesion molecules [1–3]. Ectodomain shedding has emerged as a critical post-translational modulator of the cleaved substrate proteins, as it can activate, inactivate, or change the properties of the processed protein. For example, lipids of the epidermal growth factor receptor usually require proteolysis for their activation (reviewed in [1]), whereas shedding of CD23 can convert a membrane-anchored receptor with protective roles in asthma into a soluble form which can exacerbate an allergic response (see [4], and references therein). Because of the role of ADAMs and ectodomain shedding in development and disease, and since several ADAMs have been reported to be dysregulated in diseases such as cancer and arthritis, it is important to learn more about the catalytic properties of different ADAMs, and to identify potential substrates.

This study is focused on ADAM15, a widely expressed ADAM, which has been implicated in pathological neovascularization and in arthritis [5–10]. Human ADAM15 is located on chromosome 1 at 1q21.3, and thus in a region known to be amplified in cancers such as metastatic prostate cancer, breast cancer and melanoma [11–15]. Moreover, recent studies have provided evidence for a role of ADAM15 in prostate cancer metastasis and in breast cancer [16–18]. ADAM15 contains the catalytic site consensus sequence HEXXH [9,10], and was recently reported to process E-cadherin [19], yet little is known about its catalytic properties in biochemical assays or its response to activators and inhibitors of metzalloproteinases in cells. Although a decrease in E-cadherin shedding was observed in shRNA (small hairpin RNA)-treated MCF7 cells [19], loss-of-function studies in mouse embryonic fibroblasts (MEFs) lacking ADAM15 showed no defects in E-cadherin shedding [20]. Moreover, no defects in the shedding of several other substrates have been observed in Adam15−/− cells to date (see, for example, [4,21–26]). In order to identify a robust assay for the catalytic activity of ADAM15, we purified recombinantly expressed ADAM15 for biochemical studies. We also used overexpression of full-length membrane-anchored ADAM15 together with several alkaline phosphatase (AP)-tagged membrane proteins in cell-based assays to identify potential substrates of ADAM15. Only one of the substrates tested here, the FGFR2iiib (fibroblast growth factor receptor 2iiib), which was identified previously as a substrate for ADAM9 [27], was shed by ADAM15, thereby providing an assay for characterizing the catalytic properties of ADAM15, referred to as its catalytic ‘fingerprint’, in cell-based assays [28].
EXPERIMENTAL

Cell lines and reagents

Simian virus large T-antigen-immortalized mouse *Adam15*−/− fibroblast cell lines and respective wild-type cells were generated and characterized as described previously [21]. COS-7 cells were obtained from the American Type Culture Collection. All cell lines were grown in Dulbecco’s modified Eagle medium supplemented with antibiotics and 5% fetal calf serum. *Drosophila* SL3 cells were grown in SF900II (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 1% fetal calf serum at 27°C. All the reagents were from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless otherwise indicated. Ionomycin and the hydroxamate inhibitors GM6001 and TAPI-2 were obtained from Calbiochem (San Diego, CA, U.S.A.). Marimastat was synthesized using a slightly modified version of the two previously described procedures [29,30]. Anti-ADAM15 antibodies were generated and characterized as described previously [31]. TIMP-1 (tissue inhibitor of metalloproteinases 1) and TIMP-2 were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Full-length human TIMP-3 was a gift from Dr R. Black (Amgen, Seattle, WA, U.S.A.).

Expression vectors

The expression vectors for ADAM15 and plasminoids encoding AP-tagged epidermal growth factor receptor ligands, TNFα (tumour necrosis factor α), Kitl-1 and FGFR2iiib have been described previously [9,23,27,31–33]. The expression vector encoding Streptagged human ADAM15 for biochemical purification was cloned by inserting the ectodomain (amino acids 29–692) of human ADAM15 into the insect cell expression vector pMT-BIP/Strep-tagged human ADAM15 for biochemical purification was previously [9,23,27,31–33]. The expression vector encoding necrosis factor

transfected cell pools. For expression of ADAM15, cells were transfected with 500 μg/ml hygromycin added to the cells. One day later, the medium was exchanged for a 10-fold dilution with 0.5 ml of SF900II medium, the liposomes were incorporated into the cells. After 2 days, the medium was exchanged for a 10-fold dilution with 0.5 ml of SF900II medium containing 500 μg/ml hygromycin. The expression vectors from glutamate to alanine (E349A) were performed with the Stratagene Quick Change kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer’s recommendations.

Site-directed mutagenesis

Site-directed mutagenesis of the catalytic site of ADAM15 in expression vectors from glutamate to alanine (E349A) were performed with the Stratagene Quick Change kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer’s recommendations.

Overexpression, purification and refolding of recombinant ADAM15

Expression constructs were co-transfected with the selection vector pCoHygro (Invitrogen, Carlsbad, CA, U.S.A.) into *Drosophila* SL3 cells (6 × 10⁶ cells/ml/well in a 24-well plate) using Cellfectin (Invitrogen, Carlsbad, CA, U.S.A.). Liposomes were formed by incubating 10 μl of Cellfectin, 1 μg of the expression plasmid, and 0.1 μg of the pCoHygro vector for 15 min at room temperature in a total volume of 100 μl. After dilution with 0.5 ml of SF900II medium, the liposomes were added to the cells. One day later, the medium was exchanged against 1 ml of SF900II medium plus 1% FCS and 2 days after transfection, cells were cultured with 500 mg/ml hygromycin B (Invitrogen, Carlsbad, CA, U.S.A.) for 4 weeks to get stably transfected cell pools. For expression of ADAM15, cells were incubated with medium containing 200 μM ZnCl₂ and 10 μM CdCl₂ in a 10-litre BioWave reactor for 72 h. Two litres of the conditioned supernatants were harvested, concentrated (10-fold) and centrifuged at 40 000 g for 30 min. Recombinant enzymes were purified from the supernatant of stably expressing *Drosophila* SL3 cells. The samples were applied to a 25-ml Streptactin Sepharose (Westburg, Leusden, The Netherlands) XK 16 column which was equilibrated with 100 mM Tris/HCl (pH 7.0), containing 1 mM CaCl₂ and 10 μM ZnCl₂. The column was washed with the above buffer and the protein was eluted with 100 mM Tris/HCl (pH 7.0), 1 mM CaCl₂ and 10 μM ZnCl₂ containing 3 mM D-desthiobiotin. Peak fractions were analysed by SDS–PAGE, pooled, and concentrated with centrifugal filter devices (Amicon Biomax UFV4BCC25 4ml, Millipore, Bedford, MA, U.S.A.). The concentrated protein was applied to a size-exclusion chromatography column (Superdex 200, HiLoad 16/60, GE Healthcare, Glattbrugg, Switzerland), which was equilibrated with 100 mM Hepes (pH 7.0) containing 1 mM CaCl₂ and 10 μM ZnCl₂, at a flow rate of 1 ml/min. Fractions containing ADAM15 were pooled and concentrated with centrifugal filter devices (Amicon Biomax UFV4BCC25 4ml, Millipore, Bedford, MA, U.S.A.) to a volume of 6.6 ml containing 2.4 mg of ADAM15.

Substrate screening on peptide library

A library comprising 360 octamer peptides with different primary sequences was used to screen for a suitable substrate for a biochemical characterization of recombinant ADAM15. To enable the detection of a successful cleavage event by a simple fluorescence intensity measurement, the peptides were double-labelled with the quencher DABCYL [4-(4-dimethylaminophenylazo)benzoic acid] at the N-terminus and the fluorophore EDANS {5-[(2-aminoethyl)amino]napththalene-1-sulphonic acid} at the C-terminus of each peptide. The library (protease substrate set PrSS-360-75) was purchased from JPT Peptide Technologies (Berlin, Germany); a complete list of screened peptides can be found on the manufacturer’s homepage, http://jpt.com/index.htm). The peptides were delivered as dry powders. According to the supplier, the purity of the individual preparation was ≥95% as determined by high-performance liquid chromatography. The powders were dissolved in dry dimethyl sulfoxide and kept as 5 mM stock solutions at −20°C.

Prior to the screening experiment, the stock solutions of the enzyme preparations and those of the peptides were diluted with ‘assay buffer’, comprising 100 mM Hepes (pH 7.0), containing 1 mM CaCl₂, 10 mM ZnCl₂, 0.05% (w/v) CHAPS [3-[(3-cholamidopropyl)-dimethylammonio]-propanesulfonate]. The individual solutions were transferred to 384-well plates (Black Microtiter 384 Plate, round well; Cat. No. 95040020 Thermo Electron, Oy, Finland) by means of a CyBi-Well 96-channel pipettor (CyBio AG, Jena, Germany). The final assay concentrations were 600 nM for the enzyme and 10 μM for the dual-labelled peptides. Fluorescence measurements were conducted using an Ultra Evolution fluorescence plate reader (TECAN, Maennedorf, Switzerland). The instrument was equipped with 350 nm (20 nm bandwidth) and 500 nm (25 nm bandwidth) bandpass filters for fluorescence excitation and emission acquisition, respectively. To increase the signal-to-background ratio, a dichroic mirror with a cut-off wavelength at 410 nm was used. The fluorescence intensity of each well was averaged over three flashes per measurement.

Cell culture, transfection and ectodomain shedding assay

Fibroblasts were transfected with Lipofectamine2000, while COS-7 cells were transfected with Lipofectamine as described [23,25], using the indicated plasmids. Shedding assays were performed the day after transfection. For individual shedding experiments, the cells were washed with OptiMEM medium, which was replaced after 1 h by fresh OptiMEM with or without the indicated inhibitors or stimuli, and incubated for 1–4 h (see legends of individual figures) as described [23]. Evaluation of AP activity in the supernatant and cell lysates by SDS–PAGE or by colorimetric assays was performed as described previously [23,34]. No AP activity was present in conditioned media of non-transfected cells. Three identical wells were prepared, and the ratio between the total AP activity in the supernatant and the total
AP activity in the cell lysate plus supernatant were calculated for normalization. The ratio reflects the relative shedding activity of a given sheddase towards a given AP-tagged ligand. The same wells were used to collect supernatants after 3 h of constitutive shedding and 3 h of shedding in the presence of inhibitors. Western blot analysis for ADAM15 was performed as described previously [9,31].

**Statistical analysis**

All values are expressed as means ± S.E.M. The standard error values indicate the variation between mean values obtained from at least three independent experiments. The assumptions for normality (Kolmogorov–Smirnov test) and equal variance (Levene median test) were verified with SigmaStat 3.1 software (Erkrath, SYSSTAT, Germany). One-way analysis of variance was performed. Multiple parametric statistical comparisons between experimental groups versus a control group were accomplished by Dunnett’s method. P values of < 0.05 were classified as statistically significant.

**RESULTS**

**Purification of soluble ADAM15 for biochemical assays of its catalytic activity**

In order to learn more about the catalytic properties and inhibitor profile of ADAM15, we expressed its soluble ectodomain in *Drosophila* SL3 cells, and purified it using affinity chromatography in combination with gel filtration. As control, we isolated a mutant form of the soluble ADAM15 ectodomain containing an inactivating HEXXH → HAXXH (E → A) mutation in its catalytic site, which was expressed and purified under identical conditions as the wild-type enzyme. A Coomassie Brilliant Blue-stained gel of the purified proteins is shown in Supplementary Figure S1 (at http://www.BiochemJ.org/bj/420/Suppl/add.htm). However, although previous studies with other ADAMs, including ADAM8 [35], ADAM9 [36], ADAM10 [37], ADAM12 [38], ADAM17 [39,40], ADAM19 [41] and ADAM28 [42] all were able to identify at least one peptide substrate for the recombinantly expressed ADAM, no catalytic activity of the highly purified recombinant soluble ADAM15 ectodomain was detected towards various peptide substrates, represented in a peptide library that is commonly used for unbiased substrate identification (see Experimental Procedures for details). In addition, myelin basic protein and the insulin B chain were tested for cleavage by ADAM15; however, these proteins were not also specifically cleaved by ADAM15.

**Full-length membrane-anchored ADAM15 cleaves AP-tagged FGFR2iiib in cell-based assays**

As an alternative to the enzymatic study of the catalytic activity of purified human ADAM15, we tested whether full-length membrane-anchored ADAM15 had the capacity to shed the ectodomain of various membrane proteins when overexpressed in cells, since several other ADAMs tested to date also had activity towards one or more substrates in cell-based assays when compared with their catalytically inactive control [21,27]. We therefore performed ‘gain-of-function’ overexpression studies, in which wild-type mouse ADAM15 or the catalytically inactive ADAM15E→A mutant were transfected together with one of several AP-tagged membrane proteins in mEFs. Overexpression of ADAM15 had no effect on release of AP-tagged amphiregulin (Figure 1A), β-cellulin (Figure 1B), epiregulin (Figure 1C) Kit-l (Figure 1E), transforming growth factor α (TGFα) (Figure 1F), TNFα (Figure 1G), or TRANCE (TNF-related activation-induced cytokine) (Figure 1H), but increased the shedding of FGFR2iiib (Figure 1D) compared with cells overexpressing the inactive ADAM15E→A mutant. The insets in each panel show an SDS–PAGE of the AP-tagged substrate in the cell lysate and cell supernatant, as visualized by in-gel AP staining [34]. In the case of FGFR2iiib, there was less AP-tagged FGFR2iiib in the lysates of cells expressing ADAM15 compared with cells expressing the 15 E→A control, and more of the soluble form was released into the supernatant of ADAM15-expressing cells compared with control cells. This demonstrates that the membrane-anchored mature form of FGFR2iiib, which is the slower migrating form of the AP-tagged FGFR2iiib (marked by an asterisk in Figure 1D), is processed and largely consumed when ADAM15 is overexpressed, resulting in increased release of the soluble ectodomain into the supernatant.

To further corroborate that ADAM15 can process and shed FGFR2iiib in different cell types, we overexpressed ADAM15 or the E→A mutant with FGFR2iiib–AP in COS-7 cells (Figure 2A) or in Adam15−/− mEFs (Figure 2B). In both cases, overexpression of ADAM15 reproducibly increased FGFR2iiib shedding by approx. 50% compared with cells overexpressing the inactive ADAM15E→A. Western blot analysis showed that the E→A control was expressed at higher levels than wild-type ADAM15 in COS-7 cells, and at similar levels in Adam15−/− mEFs (Figures 2A an 2B; lower panels). Moreover, an in-gel assay of AP activity corroborated both the increase in FGFR2iiib in the culture supernatant and the decrease in the cell lysate of COS-7 cells or Adam15−/− mEFs expressing ADAM15 compared with controls expressing ADAM15E→A (Figures 2A and 2B; lower panels). The ADAM15-dependent shedding of FGFR2iiib could be further enhanced by increasing the amount of ADAM15 plasmid used to transfect cells (Figure 2C). Since introducing an E→A mutation in the catalytic site of other ADAMs is known to abolish their catalytic activity [21,36,43,44], these experiments confirm that overexpression of wild-type ADAM15 increases shedding of FGFR2iiib compared with the inactive control, thereby providing a robust readout for the catalytic activity of ADAM15 in several different cell lines. However, when up to 50 μg of purified recombinant soluble ADAM15 (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/420/bj4200105add.htm) was added to FGFR2iiib expressing COS-7 cells, no increase in the release of the soluble FGFR2iiib ectodomain was observed (Supplementary Figure S2 at http://www.BiochemJ.org/bj/420/bj4200105add.htm). Therefore the shedding of FGFR2iiib could only be used to assess the catalytic properties of overexpressed membrane-anchored ADAM15, but not of the soluble purified metalloproteinase.

**Effect of hydroxamate-type metalloproteinase inhibitors and TIMPs on ADAM15-mediated FGFR2iiib shedding**

ADAMs and related metzincin metalloproteinases can usually be inhibited by hydroxamic acid-type metalloproteinase inhibitors [28]. Thus, we examined whether the ADAM15-mediated shedding of FGFR2iiib was sensitive to this type of inhibitors. In order to measure the effect of inhibitors or activators of shedding on ADAM15 versus the endogenous sheddase(s) for FGFR2iiib (which remains to be identified), we subtracted the AP activity on ADAM15-mediated FGFR2iiib shedding from at least three independent experiments. The assumptions of the statistical analyses were verified with SigmaStat 3.1 software. One-way analysis of variance was performed. Multiple parametric statistical comparisons between experimental groups versus a control group were accomplished by Dunnett’s method. P values of < 0.05 were classified as statistically significant.
To identify a cell-based assay for the catalytic activity of ADAM15, mouse ADAM15 or an inactive ADAM15E→A control, in which the catalytic site consensus sequence HEXXH was mutated to HAXXH, were expressed together with different candidate substrates in wild-type mEFs. All substrates were tagged with an AP moiety to allow detection of the cleaved ectodomain in the cell supernatant. The effect of overexpression of ADAM15 on the release of amphiregulin (A), betacellulin (B), epiregulin (C), FGFR2iiib (D), Kitl-1 (E), TGFα (F), TNFα (G) and TRANCE/OPGL (H) is depicted as the AP activity released into the culture supernatant, with the release of AP from cells expressing the ADAM15E→A mutant set to 1, and used as a reference point in each experiment. Shedding of FGFR2iiib (D) was increased approx. two-fold in cells expressing ADAM15 compared with cells overexpressing the inactive ADAM15E→A mutant. The insets in each panel show the AP-tagged substrate in the cell lysate (CL) and cell supernatant (SN), as visualized by in-gel AP staining [23,34]. The slower migrating form of the FGFR2iiib in the lysate of cells expressing ADAM15E→A that is not present in cells expressing ADAM15 is marked by an asterisk in the inset. Each panel is representative of at least three separate experiments. *In the graph in panel D the asterisk denotes P < 0.05.

0.17 μM for marimastat, 0.24 μM for GM6001 and 8.2 μM for TAPI-2. In addition, we tested whether TIMP-1, TIMP-2 and TIMP-3 affected the shedding of FGFR2iiib by ADAM15. TIMP-1, TIMP-2 and TIMP-3 are known to inhibit most matrix metalloproteinases [45], and TIMP-3 is a potent inhibitor of ADAM10 and ADAM17, whereas TIMP-1 inhibits ADAM10, but not ADAM17 [21,46–48]. When TIMP-1, TIMP-2 or TIMP-3 were added to cells overexpressing FGFR2iiib–AP together with ADAM15 or its inactive E→A mutant, the increase in shedding seen in cells overexpressing ADAM15 compared with cells expressing the E→A control was not significantly affected by concentrations of TIMP-1 up to 100 nM (Figure 4A), and only weakly blocked by 100 nM of TIMP-2 (Figure 4B), whereas TIMP-3 inhibited ADAM15 partially at 20 nM, and strongly at 50 nM, with complete inhibition observed at 100 nM (Figure 4C).

The ADAM15-dependent FGFR2iiib shedding is not stimulated by PMA or calcium ionophores

Shedding of membrane proteins is known to occur in a constitutive and regulated fashion [1]. For example, the phorbol
Figure 2  Overexpression of ADAM15 increases shedding of FGFR2iiib in COS-7 cells and ADAM15-deficient mEFs

Overexpression of ADAM15 or the catalytically inactive ADAM15E→A mutant (1.5 μg each) with FGFR2iiib in COS-7 cells (A) or in Adam15−/− cells (B). In both cases, there was a significant increase in FGFR2iiib shedding from cells overexpressing wild-type ADAM15 compared with cells overexpressing ADAM15E→A. Western blot analysis confirmed overexpression of ADAM15 or the E→A control at similar levels, and an in-gel detection of the AP-tagged FGFR2iiib shown in the lower panels provides independent verification of the colorimetric results depicted in the graphs in the upper panels. SN, cell supernatant; CL, cell lysate. (C) Increasing amounts of transfected ADAM15 cDNA increased the shedding of co-transfected FGFR2iiib from COS-7 cells. Each panel is representative of at least three separate experiments. *P < 0.05.

ester phorbol 12-myristate 13-acetate (PMA) is frequently used to stimulate ADAM17-dependent ectodomain shedding, whereas the calcium ionophore ionomycin can activate shedding by ADAM10 and ADAM17 [21,48–50]. When we tested how 20 ng/ml PMA affects ADAM15-dependent shedding of FGFR2iiib–AP in COS-7 cells, we found that the small increase in shedding in the presence of PMA was similar to that seen in the cells expressing ADAM15E→A (Figure 5A) or in Adam15−/− mEFs or COS-7 cells transfected only with FGFR2iiib (Supplementary Figures S3A and S3B at http://www.BiochemJ.org/bj/420/bj4200105add.htm). These results suggest that ADAM15, as well as the endogenous sheddase(s) for FGFR2iiib–AP, are not stimulated by 20 ng/ml PMA. When identical experiments were performed using 2.5 μM ionomycin to stimulate shedding, we observed a strong increase in FGFR2iiib–AP shedding from COS-7 cells transfected with the ADAM15E→A control (Figure 5B) as well as from Adam15−/− mEFs and COS-7 cells expressing only FGFR2iiib–AP (Supplementary Figures S3A and S3B at
The catalytic activity of ADAM15 in cell-based assays is sensitive to the hydroxamates marimastat, GM6001 and TAPI-2. The effects of different concentrations of the hydroxamates marimastat (A), GM6001 (B), and TAPI-2 (C) on the increase in FGFR2iiib shedding in cells overexpressing ADAM15 compared with cells expressing ADAM15E→A are shown. Each panel is representative of at least three separate experiments.

Figure 3

http://www.BiochemJ.org/bj/420/bj4200105add.htm), indicating that the endogenous sheddase(s) for FGFR2iiib–AP responds strongly to this concentration of ionomycin, but not to PMA. When wild-type ADAM15 was overexpressed in COS-7 cells together with FGFR2iiib–AP, the overall levels of shedding in the presence or absence of ionomycin increased, however, the absolute increase in shedding in the presence of ionomycin was similar in cells expressing ADAM15E→A or ADAM15. These results indicate that ADAM15 also does not respond to stimulation with ionomycin, at least under the conditions tested here.

DISCUSSION

ADAM15 is a widely expressed metalloproteinase disintegrin which has been implicated in pathological neovascularization and

Figure 4

TIMP-inhibitor profile of ADAM15 in cell-based assays

The effect of 20, 50 or 100 nM TIMP-1 (A), TIMP-2 (B), and TIMP-3 (C) was tested on cells expressing FGFR2iiib together with ADAM15 or catalytically inactive ADAM15E→A. The numbers in each bar indicate the percentage shedding under different conditions, using untreated cells expressing ADAM15E→A as a reference for background shedding, which is set to 100 %. The numbers above the arrows represent the increase in shedding of FGFR2iiib by ADAM15 relative to the endogenous sheddase in cells expressing FGFR2iiib and ADAM15E→A, in the presence or absence of different concentrations of TIMPs. TIMP-1 had no significant effect on background shedding in the ADAM15E→A-expressing cells. In cells expressing ADAM15, there was an increase in FGFR2iiib shedding of approximately 50 %, and this was not significantly affected by up to 100 nM TIMP-1. Concentrations of up to 50 nM TIMP-2 also did not affect FGFR2iiib background shedding in the presence of ADAM15E→A, while 100 nM led to a small decrease. The stimulation of FGFR2iiib shedding by ADAM15 was also not significantly affected by up to 50 nM TIMP-2, but was slightly decreased in the presence of 100 nM TIMP-2. The effects of TIMP-3 appeared to be similar on ADAM15 and the endogenous sheddase, in that 20 nM and 50 nM partially blocked FGFR2iiib shedding by both activities, whereas 100 nM TIMP-3 reduced ADAM15-dependent shedding to background levels seen in cell expressing ADAM15E→A. These results are representative of at least 3 separate experiments. *P < 0.05, the effect of TIMPs on the endogenous FGFR2iiib sheddase. +P < 0.05, the effect of TIMPs on shedding of FGFR2iiib by overexpressed ADAM15.
control allowed a determination of the inhibitor profile of ADAM15 for three hydroxamic acid-type metalloproteinase inhibitors, marimastat, TAPI-2, and GM6001. Regarding the use of ADAM15E→A as an inactive control, one concern was that this could potentially act on FGFR2iiib shedding in a dominant negative manner. However, we did not observe any difference in shedding from cells co-transfected only with FGFR2iiib (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/420/bj4120105add.htm) compared with cells co-transfected with FGFR2iiib and ADAM15E→A, which rules out potential dominant negative effects of ADAM15E→A in this assay.

When we tested how ADAM15-dependent processing of FGFR2iiib responds to TIMPs, we found no effect of up to 100 nM TIMP-1, only a weak effect of 100 nM TIMP-2, but significant inhibition by 20 nM and 50 nM TIMP-3, and complete inhibition by 100 nM TIMP-3 in cell-based assays, providing the first information on the TIMP inhibitor profile of ADAM15. The lack of substantial inhibition of ADAM15-dependent processing of FGFR2iiib by TIMP-1 and TIMP-2 strongly suggests that ADAM15 acts directly on FGFR2iiib, instead of indirectly, for example via activation of a matrix metalloproteinase, as TIMPs-1 and -2 are able to inhibit most, if not all matrix metalloproteinases [45]. Moreover, we found that overexpressed ADAM15 did not respond to stimulation with phorbol esters or calcium ionophores, two commonly used stimuli of ectodomain shedding. These properties distinguish ADAM15 from ADAM10 and ADAM17 in that ADAM10- or ADAM17-dependent shedding can be post-translationally upregulated by phorbol esters (ADAM17) or ionomycin (ADAM10 and 17) within 30 min [21]. Instead, we found that transfections with increasing amounts of ADAM15 also increased the processing of FGFR2iiib (see Figure 2C), suggesting that ADAM15 is constitutively active, with increased expression levels corresponding to increased activity. Therefore it appears likely that strongly increased and dysregulated expression of ADAM15 under pathological conditions, such as in neovascular tufts in the retina of mice subjected to a mouse model for oxygen induced retinopathy [8], also correlates with increased ADAM15-dependent processing of its substrates in these cells. However, these results do not rule out the possibility that the activity of ADAM15 might be further enhanced by other yet to be identified stimuli.

The characterization of the catalytic properties of ADAM15 in cell-based assays describes a ‘fingerprint’ of ADAM15 by providing information on its characteristic response to the commonly employed inhibitors and activators of proteolysis tested here. This information can be utilized to test whether or not the catalytic activity of ADAM15 could be relevant for substrate processing in specific cell types or in human disease. For example, shedding of a substrate, which can be blocked at the concentrations of the hydroxamates used here and by 100 nM TIMP-3, but not by 100 nM TIMP-1, and only weakly by 100 nM TIMP-2, and is not activated by PMA or ionomycin, would raise the possibility that ADAM15 is the relevant sheddase. This hypothesis could then be subjected to further validation using cells or tissues from Adam15<sup>−/−</sup> mice, or cells treated with ADAM15 shRNA. In addition, the identification of the release of FGFR2iiib as a robust assay for the catalytic activity of ADAM15 provides a means of identifying novel inhibitors of the catalytic activity of ADAM15, which might be useful to treat breast cancer patients with overexpressed or dysregulated ADAM15 [16], or prevent ADAM15-dependent metastasis [17] or pathological neovascularization and proliferative retinopathy [8].

It remains to be determined why biochemically purified ADAM15 did not display catalytic activity towards any of the

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**Figure 5** Stimulation of ADAM15 in cell-based assays

The effect of 20 ng/ml PMA (A) or 2.5 μM ionomycin (B) on ADAM15-dependent shedding of FGFR2iiib. The numbers in each bar represent the percentage shedding compared with cells expressing ADAM15E→A, where shedding in the absence of treatment was used as a reference, and set to 100 %. (A) Treatment with PMA had very little effect on FGFR2iiib shedding in the presence or absence of active ADAM15. (B) Treatment with ionomycin strongly increased shedding of FGFR2iiib (by approx. 275 %) from cells expressing ADAM15E→A, so this effect was due to one or more endogenous sheddases. A similar increase in shedding (approx. 300 %) was seen in cells expressing ADAM15, suggesting that ADAM15 is not significantly stimulated by ionomycin. These results are representative of at least three separate experiments.

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in tumorigenesis [8,17–19]. Yet, even though ADAM15 carries a catalytic site consensus sequence, little is known about its catalytic properties in cells, including its response to activators or inhibitors of proteolysis, also referred to as its catalytic ‘fingerprint’ [28]. Here we describe a robust cell-based assay for the catalytic activity of full-length membrane-anchored ADAM15, the increase in shedding of FGFR2iiib by overexpressed ADAM15 compared with the overexpressed catalytically inactive ADAM15E→A mutant. The activity of ADAM15 was reflected both in the increased shedding of soluble FGFR2iiib, and in the decrease in the cell-associated form of this receptor. While the physiological relevance of ADAM15 in processing the FGFR2iiib remains to be determined, it is tempting to speculate that ADAM15 could contribute to the down-regulation of FGFR2iiib, at least in cells or tissues in which both proteins are highly expressed. However, for the purpose of the present study, shedding of FGFR2iiib only served as a readout for characterization of the catalytic activity of ADAM15 in cells, and thereby provided an alternative to biochemical assays with highly purified recombinant soluble ADAM15, which failed to process any of the large number of peptide substrates tested in this study.

ADAM15-dependent FGFR2iiib shedding compared with shedding from cells expressing the inactive ADAM15E→A 
substrates tested here, despite its high purity and concentration. The only weak activities that were occasionally observed were interpreted as contaminants because they were also present in the purified ADAM15E-A mutant, which is a critical control to rule out such activities. Interestingly, purification of other recombinantly expressed ADAMs has also shown that these proteins rapidly lose activity during purification, unless the enzyme is stabilized by incubation in empirically determined buffer conditions [44]. Therefore it is possible that recombinantly expressed ADAM15 also lost activity during purification, despite repeated attempts to conserve its activity using different buffers. An alternative interpretation for the lack of catalytic activity of ADAM15 in biochemical assays is that its substrate selectivity could be more restricted than that of other ADAMs, all of which were able to process one or more peptide substrates, and/or the insulin-B chain or myelin basic protein [35,36,39–42,44,51]. Finally, it is possible that ADAM15 requires expression in the context of intact cells to display catalytic activity.

In summary, this study presents a robust and highly reproducible cell-based assay for the catalytic activity of ADAM15. In light of the various medically relevant functions that have emerged for ADAM15, including roles in pathological neovascularization, breast cancer and prostate cancer metastasis, this assay provides novel insights into the catalytic properties of ADAM15 in the context of intact cells, which will allow screening for inhibitors to block detrimental functions of this widely expressed enzyme.

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SUPPLEMENTARY ONLINE DATA
Characterization of the catalytic activity of the membrane-anchored metalloproteinase ADAM15 in cell-based assays

Thorsten MARETZKY*, Guangli YANG†, Ouathek OUERFELLI‡, Christopher M. OVERALL‡§, Susanne WORPENBERG∥, Ulrich HASSIEPEN∥, Joerg EDER∥ and Carl P. BLOBEL*†

*Arthritis and Tissue Degeneration Program, Hospital for Special Surgery at Weill Medical College of Cornell University, 535 E 70th Street, New York, NY 10021, U.S.A., †Memorial Sloan-Kettering Cancer Center, New York, NY 10065, U.S.A., ‡Department of Oral Biological and Medical Sciences, University of British Columbia, Vancouver, BC, Canada, V6T 1Z3, §Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada, V6T 1Z3, and ∥Center for Proteomic Chemistry, Novartis Institutes for Biomedical Research, CH-4002 Basel, Switzerland

Figure S1 Purified recombinant extracellular domains of ADAM15 and the inactive ADAM15E→A variant

Equal amounts of the purified ectodomains of soluble ADAM15 or ADAM15E→A were separated by SDS–PAGE and stained with Coomassie Brilliant Blue. However, in tests for the processing of peptide substrates, no activity of the ADAM15 ectodomain towards 360 different peptide substrates was identified (see Experimental Procedures and Results sections for details).

Figure S2 Incubation of COS-7 cells expressing FGFR2iiib–AP with increasing concentrations of the purified soluble recombinant extracellular domain of ADAM15

Increasing concentrations of purified soluble recombinant ADAM15 added to FGFR2iiib-expressing COS-7 cells had no effect on the release of the soluble ectodomain of FGFR2iiib.

Figure S3 Shedding of FGFR2iiib–AP from Adam15−/− cells or COS-7 cells in the presence of PMA or ionomycin

Adam15−/− cells (A) or COS-7 cells (B) were transfected with FGFR2iiib–AP and then stimulated with 20 ng/ml PMA or 2.5 μM ionomycin (IM) for 30 min to characterize the response of the endogenous sheddase for FGFR2iiib to these stimuli in these two cell types. In both cell types, the endogenous sheddase responded to stimulation with IM, but not with PMA.

1 To whom correspondence should be addressed: email blobelc@hss.edu