

The use of acumen[®] eX3 for the identification of the roles of Protein Kinase C α and δ isoforms in PMA-induced HB-EGF ectodomain shedding.

EGFR ligands and their role in disease

EGFR ligands are a family of proteins which are involved in the regulation of cell growth and differentiation and their over-expression is associated with a number of cancers, including breast, lung and colon cancer. These ligands are expressed on the cell surface and include proteins such as epidermal growth factor (EGF), heparin binding EGF (HB-EGF) and transforming growth factor α (TGF α).

Proteolytic cleavage of the transmembrane form of these ligands from the plasma membrane enables these ligands to bind to EGFR and activate signalling pathways involved in cell migration, adhesion and proliferation. The release of mature receptor ligands from their membrane-anchored precursor form is a process termed “ectodomain shedding” and is a highly regulated process that can be stimulated by events such as calcium influx or protein kinase C (PKC) activation. Although the underlying mechanism of EGFR ligand shedding remains poorly understood, it appears that several members of the ADAM (a disintegrin and metallo-proteinase) family have been implicated in the activated shedding of EGFR ligands. In a recent study, Le Gall et al. [1] demonstrated that ADAM17 is one of the principal enzymes that respond to the stimulation of a number of signalling pathways involved in EGFR ligand shedding. Indeed earlier studies show ADAM17 to be the major Phorbol 12-Myristate 13-Acetate (PMA)-responsive sheddase, cleaving Pro-HB-EFG to produce bioactive HB-EGF [2]. Over-expression of EGFRs is associated with a number of cancers and the inhibition of EGFR expression is the basis for a number of therapies.

the role of acumen in the study of EGFR pathways

This application note discusses a study recently published by Kveiborg and co-workers [3] highlighting how the study of immunofluorescent labelling using an acumen[®] eX3 laser scanning imaging cytometer in combination with traditional spectrophotometric methods has enabled a more detailed understanding of the pathways involved in EGFR signalling pathways and the shedding of EGFR ligands. These researchers have employed acumen's powerful image analysis capabilities for the analysis of PMA-induced HB-EGF shedding and the effect of PKC inhibitors and RNAi mediated knockdown on HB-EGF expression. The acumen eX3 is widely used for immunofluorescence analysis of protein expression or cell cycle inhibition in oncology research. Its ability to rapidly perform whole well cell-based screening allows cell number enumeration as part of the assay. Furthermore, its triple laser system enables multiplexed assays such as the assessment of cell viability alongside cell surface markers.

In addition, acumen eX3 is ideal for screening RNAi studies as it combines whole well scanning with the ability to measure cell number as part of the assay. This gives researchers the capability to normalise responses to total cell number, eliminating the need to run a separate proliferation assay.



methods

cell lines and cell culture

In this study, the role of PKC and its isoforms on the regulation of ADAM17 mediated HB-EGF shedding was studied using a human HT1080 fibrosarcoma cell line (American Type Culture collection) transfected with a cDNA construct encoding Pro-HB-EFG fused to alkaline phosphatase (AP-HB-EFG) [3].

assay for PMA-induced AP-HB-EFG shedding

HT1080 cells stably expressing AP-HB-EFG were either, seeded in 96-well plates at a concentration of 2.5×10^4 cells/well and analysed for HB-EGF shedding after 24 h, or were reverse transfected with siRNA and used in shedding assays 72 h later. The confluent cell layer was washed twice with serum-free medium (SFM) and treated with 400 nM PMA or DMSO control for 30 mins. For treatment with various chemical inhibitors, 15 min pre-incubation with inhibitor or a control in SFM was performed prior to stimulation with or without Phorbol 12-Myristate 13-Acetate (PMA).

spectrophotometric assay for alkaline phosphatase activity

For spectrophotometric quantification of AP-HB-EGF shedding, cell-conditioned medium was harvested and mixed 1:1 with a 2 mg/mL solution of the alkaline phosphatase substrate 4-nitrophenyl in 96-well plates. The reaction was incubated at 37°C for 1 h in the dark, and alkaline phosphatase activity was measured from the absorbance at 405 nm. The remaining cell layer was washed in PBS, and either fixed in 4% formaldehyde for immunofluorescence analysis or lysed directly in 2x sample buffer for Western blot analysis.

immunofluorescence analysis

Cell surface expression of AP-HB-EGF was determined by immunofluorescent staining of membrane bound AP-HB-EGF. Analysis was carried out using an acumen eX3 as described by Keivborg et al. [3]. In brief, cells were incubated with the primary anti-alkaline phosphatase antibody, anti-PLAP (8B6), (Sigma), diluted 1:1000 in 1% BSA in PBS for 2 hrs at RT, washed in PBS and incubated with the secondary antibody, Alexa Fluor® 488-donkey anti-mouse IgG (Life Tech), 1:2000 in 1% BSA in PBS for 1 hr at RT. Following incubation cells were washed in PBS and analysed. For identification of live cells, DAPI (Life Tech), (1 ng/μL) was also added with the secondary antibody and analysed using acumen eX3.

For quantification, cells were stained in black 96-well plates (Greiner) and the plates were read in the acumen eX3 (TTP Labtech). DAPI and Alexa Fluor 488 fluorescence was detected using 405–470 nm and 500–530 nm bandpass filters, respectively. To distinguish nuclei from cellular debris and larger clumps of cells, cell counts were restricted to objects measuring 5–100 μm in both width and depth. The total cell number per well was estimated by dividing the total area of the cells by the average area of a single cell. To quantify cell-surface expression of Alexa Fluor 488 labelled AP-HB-EGF, the object size was restricted to 7–222 μm in both width and depth and additional fluorescent parameters including peak, mean and total intensity measurements of each cell were used to gate the population.

inhibition of PMA-induced shedding of HB-EGF

a) inhibition of ADAM17 activation

For inhibition of ADAM17 regulated PMA-induced shedding, cells were pre-incubated with TAPI-2 (10 μM), a potent inhibitor of ADAM17, or a control vehicle for 15 mins prior to stimulation with or without PMA.

b) protein kinase C inhibition

A study to identify PKC isoforms which inhibited PMA-induced HB-EGF shedding was carried out using InhibitorSelect™ 96-well Protein Kinase Inhibitor Library I and II (Calbiochem).

Other inhibitors studied include the PKC inhibitor Bisindolyl-maleimide I (BIMI), the ADAM17 inhibitor TAPI-2 and the MEK inhibitor, UO126.

c) siRNA gene silencing

In order to identify the effect of knockdown of either ADAM17 expression or PKC isoforms in PMA-induced shedding of HB-EGF, the expression of ADAM17 or endogenous PKC isoforms were reduced using SMARTpool siRNAs (Dharmacon, Thermo Fisher) as described by Kveiborg et al. [3].

results

PMA-induced shedding of HB-EGF

A study of PMA-induced shedding of HB-EGF was carried out assessing the amount of released alkaline phosphatase spectrophotometrically or by measuring the residual cell surface fluorescence intensity per cell after immuno-staining of AP-HB-EGF (Fig. 1a and b).

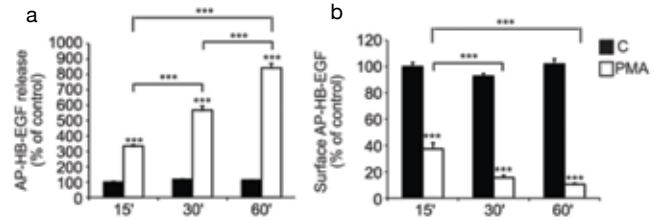


fig 1. PMA-induced ectodomain shedding of HB-EGF from the cell surface (a) AP-HB-EGF release measured as alkaline phosphatase activity (absorbance at 405 nm), (b) surface AP-HB-EGF measured as cell surface fluorescence intensity per cell in AP-HB-EGF expressing HT1080 cells after 15, 30, or 60 min treatment with 400 nM PMA (white bars) or DMSO control (black bars). All graphs show average values ± standard error of the mean of at least three independent experiments each done in triplicate. *p<0.05; **p<0.01, ***p<0.001 after one-way analysis of variance with Bonferroni's post tests for multiple comparisons. Unless otherwise indicated the comparison is relative to the respective control.

Using immunofluorescent staining of AP-HB-EGF it was observed that the treatment of cells with PMA resulted in the total release of AP-HB-EGF within 15-30 mins (Fig. 1b). Spectrophotometric analysis of AP release demonstrated a continued release of AP into the media following PMA treatment (up to 60 mins) which suggested that in the presence of PMA, newly synthesized Pro-HB-EGF continues to be shed, not remaining associated within the membrane.

These two assays complement each other by distinguishing changes in cell surface cleavage from other changes in the amount of AP-HB-EGF at the plasma membrane. The use of the acumen eX3 has enabled this research group to demonstrate the rapid release of membrane associated Pro AP-HB-EGF following exposure to PMA, confirming that Pro-HB-EGF processing and shedding in its mature form continues in the presence of PMA.

inhibition of ADAM17 and EGF shedding

In a study of the effect of the inhibition of the proteolytic activity of ADAM17 on PMA-induced shedding of Pro HB-EGF, cells were incubated with the ADAM17 inhibitor, TAPI-2. Spectrophotometric analysis of released AP shows that TAPI-2 completely prevented PMA-induced HB-EGF release (Fig. 2a). However, analysis of the fluorescence intensity of AP-HB-EGF on the membrane surface using acumen® eX3 revealed that despite complete ADAM17 inhibition, some PMA-induced loss of AP-HB-EGF was observed (Fig. 2b).

A further study of siRNA mediated ADAM17 demonstrated that loss of ADAM17 expression caused an incomplete block in PMA-induced AP-HB-EGF shedding (Fig. 2c). However, similar to that described with TAPI-2 inhibition, it was observed that ADAM knockdown did not completely inhibit the depletion of membrane bound Pro-HB-EGF (Fig. 2d)

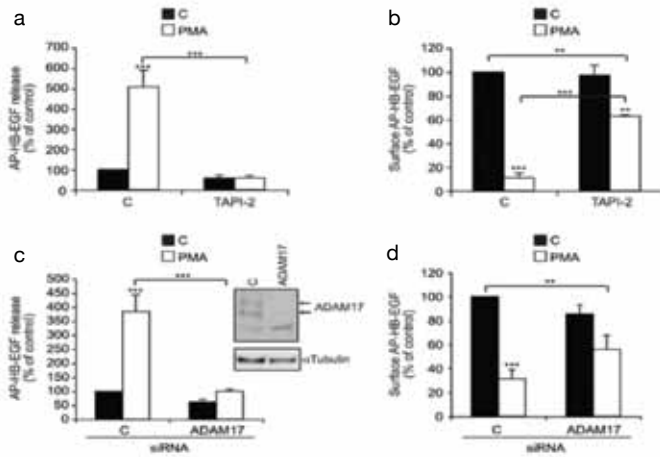


fig 2. PMA-induced HB-EGF shedding is ADAM17-dependent. (a) AP-HB-EGF release measured as alkaline phosphatase activity in conditioned media, (b) surface AP-HB-EGF measured as cell surface fluorescence intensity per cell in DMSO control-treated (C; black bars) and PMA-treated (PMA; white bars) AP-HB-EGF expressing HT1080 cells treated with a control (C) or the ADAM17 inhibitor TAPI-2. (c) and (d) show results of cell inhibition following reverse transfection with control (C) or ADAM17 siRNAs 72 h before PMA stimulation. The insert shows western blot analysis of total cell extracts from cells used in c and d, demonstrating efficient siRNA-mediated knockdown of pro and mature forms of ADAM17. α -tubulin is used as an internal loading control.

The observed loss of HB-EGF from the membrane despite inhibition of AP-HB-EGF proteolysis by ADAM17 demonstrated by immunofluorescent analysis using acumen Φ X3, suggested that there are additional factors involved in the regulation of PMA-induced ectodomain shedding of this ligand.

the role of PKC isoforms in PMA-induced HB-EGF shedding

PKC has been previously identified as a major component of the cellular signalling pathway controlling expression of Pro HB-EFG on the cell membrane surface [4] and its isoforms have been demonstrated to be involved in the stimulus coupled shedding of Pro HB-EFG [5]. As a result of this information Kveiborg et al. [3] studied the effect of PKC and its isoforms on PMA-induced HB-EGF shedding. In this study, addition of BIM1, a broad PKC inhibitor was seen to inhibit both HB-EGF cleavage and cell surface depletion in the presence of PMA (Fig. 3a and b) confirming that PKC has a direct role in the regulation of PMA-induced ectodomain shedding of HB-EGF.

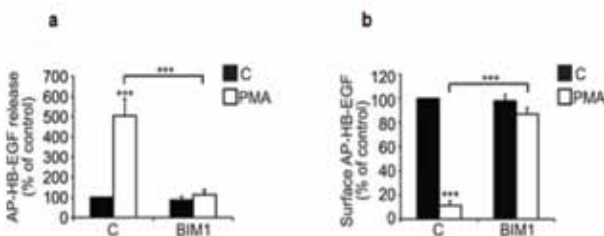


fig 3. PMA-induced HB-EGF shedding is PKC-dependent. (a) AP-HB-EGF release measured as alkaline phosphatase activity and (b) surface AP-HB-EGF measured as cell surface fluorescence intensity per cell in DMSO control-treated (C; black bars) and 30 min PMA-treated (PMA; white bars). AP-HB-EGF expressing HT1080 cells were treated with the broad PKC inhibitor BIM1 (2 μ M) or a control (C).

HB-EGF, siRNA knockdown studies specific for these PKC isoforms were carried out. Figure 4 demonstrates that knockdown of PKC α and PKC δ resulted in a statistically significant inhibition of PMA-stimulated HB-EGF shedding.

As a result of immuno-fluorescent analysis it was observed that there was a lower amount of membrane bound HB-EGF present in cells after knockdown of these isoforms, suggesting that the decreased HB-EGF shedding after knockdown may be due to a lower amount of substrate available for shedding on the cell surface rather than a reduced shedding activity (Fig. 4b).

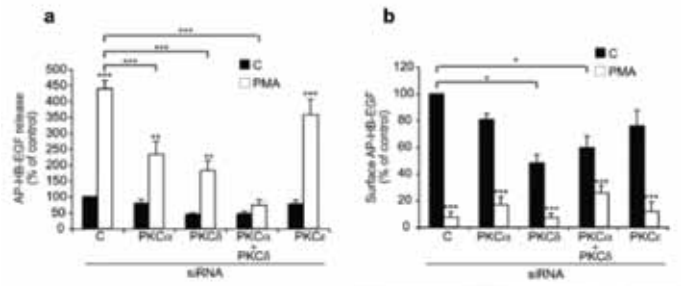


fig 4. PMA-induced HB-EGF shedding involves co-operative functions of PKC α and PKC δ . (a) AP-HB-EGF release measured as alkaline phosphatase activity, (b) surface AP-B-EGF measured as cell surface fluorescence intensity per cell in DMSO control-treated (black bars) and PMA-treated (white bars) in AP-HB-EGF expressing HT1080 cells after reverse transfection with control siRNAs (C) or siRNAs against the indicated PKC isoforms.

In addition, similar to what was observed for ADAM17 knockdown, an almost complete loss of HB-EGF shedding was not reflected by knockdown or inhibition of these PKC isoforms, in a similar complete block of cell surface removal, supporting the observation that PMA induces both HB-EGF cleavage and additional loss of cell

discussion

This study on the inhibition of PMA-induced HB-EGF shedding provides a further understanding of the role of PKC and its isoforms on EGFR signalling pathways. Employment of both the alkaline phosphatase tagged HB-EGF enzymatic assay alongside immunofluorescent labelling studies of cell surface associated HB-EGF using an acumen Φ X3 has enabled changes in cell surface cleavage to be distinguished from other changes in the amount of HB-EGF available at the plasma membrane. The ability to analyse membrane bound HB-EGF and to study the effect of the inhibition of key components involved in the process of ectodomain shedding using acumen Φ X3 enabled these researchers to demonstrate the individual roles of two specific PKC isoforms, α and δ in the signalling pathway, revealing that while PKC α specifically regulates PMA-induced shedding, PKC δ and ERK influences both constitutive and inducible shedding.

The multiplexing abilities of acumen Φ X3 enabled quantification of the presence of EGF on the total cell population hence normalising responses to total cell number. In addition, the ability of acumen Φ X3 to simultaneously obtain data and analyse the effects on PMA-induced HB-EGF shedding has provided robust data which has in turn enabled a detailed understanding of the pathways involved in EGFR signalling. Moreover, the assay developed here proves to be very suitable to high throughput screening of either arrayed compounds or siRNA collections.

This study contributes to furthering the understanding of pathways involved in EGFR signalling and may provide strategies for the inhibition of up regulation of these receptors and ligands observed in human carcinomas.

specifications

TTP Labtech's acumen® eX3 is a bench-top system which has been integrated with a wide array of other laboratory instrumentation, ranging from simple stacking robots through to complete plate preparation solutions to achieve walk-away operation in many application areas.

Detection technology:	Laser scanning imaging cytometry
Laser excitation:	Up to 3 solid state lasers in a single instrument (choice includes 405, 488, 561, 633 nm)
Detection:	4 colours simultaneously per laser using photomultiplier tubes
Resolution:	Equivalent to a 20x microscope objective
Sample format:	96, 384, 1536 and 3456 SBS-format microplates; slides
Throughput:	Typically 8 minutes regardless of plate type
File size:	Down to 50 KB per plate in HTS mode (CSV file)
File export:	Explorer® plate files, CSV, FCS and open source TIFF (8- & 16-bit)
PC operating system:	Microsoft® Windows® XP (Professional)
Configurations:	Stand-alone, self-maintained workstation or fully integrated
Laser safety:	Class 1 laser product
Dimensions:	670 mm x 504 mm x 350 mm (26 x 20 x 14") (w x d x h)
Net weight:	Approx 50 kg (110 lbs)
Services:	110/230V single phase 47/63 Hz 800W

references

- 1). Le Gall, S. M., Maretzky, T., Issuree, P. D. A., Niu, X., Reiss, K., Saftig, P., Khokha, R., Lundell, D. & Blobel, C.P. (2010) ADAM17 is regulated by a rapid and reversible mechanism that controls access to its catalytic site. *Journal of Cell Science*. 123: pp3913-22.
- 2). Horiuchi, K., Le Gall, S., Schulte, M., Yamaguchi, T., Reiss, K., Murphy, G., Toyama, Y., Hartmann, D., Saftig, P. & Blobel, C.P. (2007) Substrate selectivity of epidermal growth factor-receptor ligand sheddases and their regulation by phorbol esters and calcium influx. *Mol. Biol. Cell*. 18: pp176-88.
- 3). Kveiborg, M., Instrell, R., Rowlands, C., Howell, M. & Parker, P.J. (2011) PKC and PKC regulate ADAM17-mediated ectodomain shedding of heparin binding-EGF through separate pathways. *PLoS One*. 28: e17168.
- 4). Goishi, K., Higashiyama, S., Klagsbrun, M., Nakano, N., Umata, T., Ishikawa, M., Mekada, E. and Taniguchi, N. (1995) Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: Conversion from juxtavrine to paracrine growth factor activity. *Mol. Biol. Cell*. 6:pp967-80.
- 5). Izumi, Y., Hirata, M., Hasuwa, H., Iwamoto, R., Umata, T., Miyado, K., Tamai, Y., Kurisaki, T., Sehara-Fujisawa, A., Ohno, S. & Mekada, E. (1998) A metalloprotease-disintegrin, MDC9/meltrin-gamma/ADAM9 and PKCdelta are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. *EMBO J*. 17: pp7260-72.

TTP Labtech Ltd
Melbourn Science Park
Melbourn
Hertfordshire SG8 6EE
United Kingdom

tel: +44 1763 262626
fax: +44 1763 261964

sales@ttplabtech.com

TTP Labtech Inc
One Kendall Square
Suite B2303
Cambridge MA 02139
United States

tel: +1(617) 494 9794
fax: +1(617) 494 9795