

Ectodomain shedding of the EGF-receptor ligand epigen is mediated by ADAM17

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Abstract All ligands of the epidermal growth factor receptor (EGFR), which has important roles in development and disease, are made as transmembrane precursors. Proteolytic processing by ADAMs (a disintegrin and metalloprotease) regulates the bioavailability of several EGFR-ligands, yet little is known about the enzyme responsible for processing the recently identified EGFR ligand, epigen. Here we show that ectodomain shedding of epigen requires ADAM17, which can be stimulated by phorbol esters, phosphatase inhibitors and calcium influx. These results suggest that ADAM17 might be a good target to block the release of bioactive epigen, a highly mitogenic ligand of the EGFR which has been implicated in wound healing and cancer.

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1. Introduction

Epigen is a recently identified ligand of the epidermal growth receptor (EGFR, ErbB1), and belongs to a group of functionally and structurally similar growth factors that mediate cellular responses ranging from cell survival to proliferation and migration [6,29]. Epigen was first isolated from a mouse keratinocyte cDNA library due to its homology to the EGFR-ligand epiregulin [25]. It shows 24–37% identity to other EGFR ligands (EGF, TGF α , HB-EGF, amphiregulin, betacellulin, epiregulin), and contains six spatially conserved cysteine residues that are characteristic of members of this protein family [6]. The biological activity of recombinant epigen is similar to that of other EGFR ligands. In epithelial cells, it stimulates phosphorylation of the EGFR and downstream signaling molecules such as the mitogen activated protein kinase (MAPK), and it promotes cell proliferation in a dose-dependent manner [25].

Despite its relatively low affinity for the EGFR, epigen has an increased mitogenic potential compared to other EGFR ligands. When EGFR expressing cells were treated with equal

concentrations of EGF, TGF α or epigen, epigen was found to be the most potent mitogen [12]. Epigen effectively activates signaling via an EGFR-homodimer or through a heterodimer between EGFR and ErbB2 [12]. Epigen is widely expressed in a variety of mouse tissues, especially in multiple developing structures in the embryo, such as developing tongue papillae, the dorsal root ganglion and the inner and outer root sheath of hair follicles, all of which are regions of active proliferation [12]. Moreover, epigen expression is also observed in invasive adenocarcinomas of the breast and prostate in humans [12].

All seven EGFR ligands described to date (TGF α , HB-EGF, amphiregulin, epiregulin, EGF, betacellulin and epigen) are synthesized as membrane-anchored precursors that are subsequently released from the cell surface by proteolysis [6]. This proteolytic processing is considered to be a key regulatory step controlling the bioavailability of soluble EGFR-ligands, and thus signaling via the EGFR [18] (for a recent review, see [2]). Membrane-anchored metalloprotease-disintegrin proteins (ADAMs, a disintegrin and metalloprotease) have been implicated in the ectodomain shedding of six out of the seven currently known EGFR ligands [3,18,21,26]. Specifically, ADAM17 has been shown to have a major role in processing of TGF α , HB-EGF, amphiregulin and epiregulin, and ADAM10 is a major sheddase of EGF and betacellulin [16,18,21,22,26]. However, little is known about the enzyme(s) responsible for the proteolytic release of epigen. In this study, we show that the ectodomain of epigen is released from the cell membrane constitutively at low levels, and that its shedding can be highly upregulated by phorbol esters, calcium ionophores and tyrosine phosphatase inhibitors. Moreover, we provide evidence for a major role of ADAM17 in stimulated shedding of epigen. These results uncover a fifth EGFR-ligand as a substrate for ADAM17, an enzyme with an essential role in activating the EGFR during mouse development [18].

2. Materials and methods

An expression vector encoding the alkaline phosphatase (AP)-tagged epigen was constructed by inserting a partial cDNA for mouse epigen, which encoded for the EGF repeat, the juxtamembrane domain containing the putative cleavage site, transmembrane domain and cytoplasmic domain, into the 3' end of human placental alkaline phosphatase in the CMV-based vector APTag-5 (GenHunter Corp.). The junction between AP and epigen was placed in frame at Leu 53 at the N-terminus of the EGF repeat of mouse epigen, which has 152 amino acid residues. The epigen-AP fusion construct also contains a C-terminal Myc and His tag attached to the cytoplasmic domain of epigen.

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Wild type mouse embryonic fibroblasts (mEFs), generated from wild type E13.5 mouse embryos and cultured as described previously [28], and *Adam17*^{−/−} mEFs, which have also been described previously [19] were transfected with a cDNA vector encoding the AP-epigen fusion protein with LipofectAMINE (Invitrogen). Fresh Opti-MEM (Invitrogen) medium was added the next day, conditioned for 1 h, and then replaced with fresh medium containing one of the following: 20 ng/ml PMA (Sigma), 2 μ M ionomycin, 100 μ M pervanadate, 1 μ M batimastat (BB94, provided by D. Becherer, GlaxoSmithKline, Research Triangle Park, NC), 1–10 μ M GI254023X or GW280164X [13]. The conditioned medium containing inhibitors or activators of ectodomain shedding was collected after 1 h. Evaluation of AP activity by SDS–PAGE or spectrophotometry was performed as described previously [20].

3. Results

In order to study the ectodomain shedding of epigen from cells, an alkaline phosphatase (AP) moiety was attached to the amino terminus of the EGF repeat of epigen (Fig. 1a). A similar approach has previously been used to study ectodomain shedding of other EGFR ligands [21,28]. The main advantage of adding an AP tag is that it facilitates the detection and quantitation of shed forms of epigen. Moreover, previous studies have shown that the AP tag did not affect which enzyme was responsible for shedding other EGFR-ligands, since both the AP-tagged forms of TGF α or HB-EGF and untagged wild type forms of these EGFR-ligands were shed by ADAM17 [11,18,21,26]. When COS-7 cells were transfected with AP-tagged epigen, low levels of constitutive shedding into the culture supernatant were observed (Fig. 1b, lanes 1 and 3). Constitutive release of epigen was strongly induced after addition of the phorbol ester PMA (Fig. 1b, lane 2 and Fig. 1c). Both the constitutive and the PMA-regulated components of epigen-AP shedding could be effectively inhibited by the hydroxamate-type metalloprotease inhibitor BB94 (batimastat, Fig. 1b, lane 4 and Fig. 1c) suggesting that a metalloprotease, most likely an ADAM, is involved in this process. Specifically, the strong stimulation of epigen shedding by PMA is reminiscent of ADAM17 mediated ectodomain shedding of four other EGFR ligands, TGF α , HB-EGF, amphiregulin and epiregulin, as well as of other ADAM17-substrates such as TNF α [21,30]. Essentially identical results were obtained when these experiments were repeated in CHO cells (data not shown).

To further characterize the epigen sheddase in COS-7 cells, we tested how two distinct hydroxamate-type metalloprotease inhibitors with some selectivity against ADAM10 or ADAM17 affected epigen shedding in these cells. The hydroxamate GI254023X (GI) had previously been shown to block ADAM10-mediated constitutive release of IL6R, CX3CL1 and CXCL16 at concentrations between 1 and 10 μ M, while compound GW280264X (GW) blocked PMA induced cleavage mediated by ADAM17 at these concentrations [13]. Constitutive shedding of epigen from COS7 cells was not affected by 1 μ M GI, whereas it was effectively blocked by GW at concentrations as low as 1 μ M (Fig. 2). GI had a partial inhibitory effect on epigen shedding at 5 μ M, and increasing inhibition was observed at higher concentrations of GI (7.5 μ M and 10 μ M). However, at all concentrations tested here (1–10 μ M), GW inhibited epigen shedding more efficiently than GI. This inhibition profile suggested that ADAM17, but not ADAM10, was responsible for epigen shedding. The increased shedding of epigen following stimulation by PMA was also

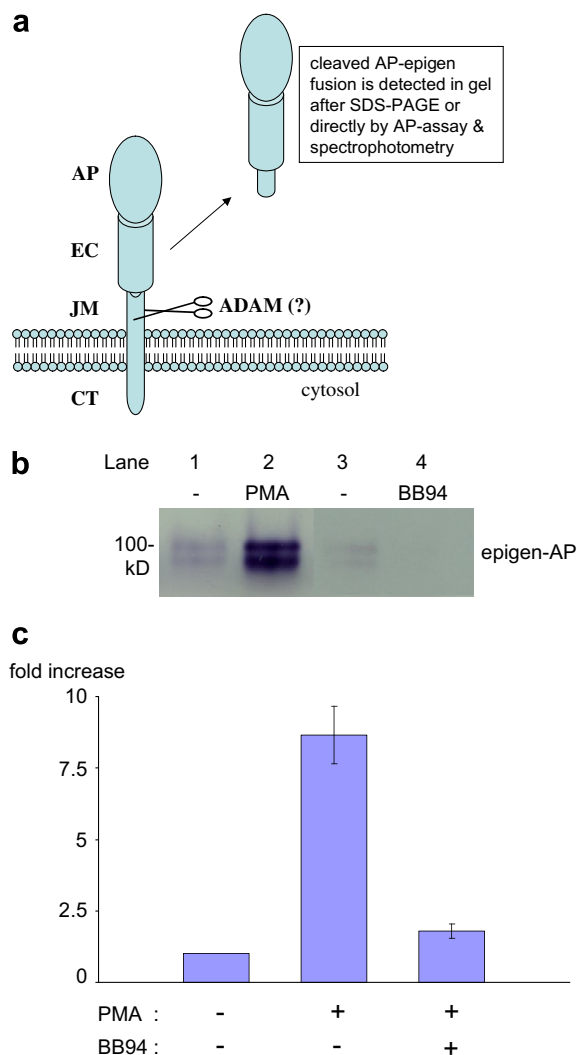


Fig. 1. Constitutive and stimulated shedding of epigen from COS-7 cells. (a) Diagram of the alkaline phosphatase (AP) reporter detection system used to monitor epigen shedding from COS-7 cells. An AP moiety was fused in frame N-terminal to the EGF-repeat (EC, extracellular domain) of mouse epigen. Proteolytic cleavage in the juxtamembrane (JM) region releases the epigen-AP fusion protein into the culture supernatant. The released epigen-AP can be detected by SDS-PAGE (b) or through spectrophotometry (c for details, see materials and methods, and reference [20]). (b) Epigen-AP is constitutively released at low levels from transiently transfected COS-7 cells (lanes 1,3). Release of epigen-AP from COS-7 cells was upregulated by 20 ng/ml PMA (lane 2) and blocked by the metalloprotease inhibitor BB94 at a concentration of 1 μ M (lane 4) (c) Upregulation of epigen-AP release from COS-7 cells upon PMA treatment is detected by a spectrophotometric assay for AP activity [20]. PMA stimulated shedding of epigen can be abolished by BB94 which also inhibits its constitutive processing. $n = 6$, error bars: standard deviation.

consistent with a role for ADAM17 in this process, since ADAM17 is considered to be one of the principal enzymes that responds to short-term stimulation with PMA (<1 h) [7,21].

In order to directly test whether ADAM17 is required for epigen shedding, we compared epigen processing in response to various stimuli in wild type mouse embryonic fibroblasts (mEF) versus mouse embryonic fibroblasts derived from *adam17*^{−/−} knockout mice (E2 cells) [18,19]. Similar to COS-7 cells, the epigen ectodomain was constitutively released from wild type mEFs at low levels, and this constitutive release

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