Extracellular Phosphorylation of Collagen XVII by Ecto-Casein Kinase 2 Inhibits Ectodomain Shedding

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Ecto-phosphorylation is emerging as an important mechanism to regulate cellular ligand interactions and signal transduction. Here we show that extracellular phosphorylation of the cell surface receptor collagen XVII regulates shedding of its ectodomain. Collagen XVII, a member of the novel family of collagenous transmembrane proteins and component of the hemidesmosomes, mediates adhesion of the epidermis to the dermis in the skin. The ectodomain is constitutively shed from the cell surface by metalloproteinases of the ADAM (a disintegrin and metalloproteinase) family, mainly by tumor necrosis factor-α converting enzyme (TACE). We used biochemical, mutagenesis, and structural modeling approaches to delineate mechanisms controlling ectodomain cleavage. A standard assay for extracellular phosphorylation, incubation of intact keratinocytes with cell-impermeable [γ-32P]ATP, led to collagen XVII labeling. This was significantly diminished by both broad-spectrum extracellular kinase inhibitors K252b and a specific casein kinase 2 (CK2) inhibitor. Collagen XVII peptides containing a putative CK2 recognition site were phosphorylated by CK2 in vitro, disclosing Ser542 and Ser544 in the ectodomain as phosphate group acceptors. Phosphorylation of Ser544 in vivo and in vitro was confirmed by immunoblotting of epidermis and HaCaT keratinocyte extracts with phosphoepitope-specific antibodies. Functionally, inhibition of CK2 kinase activity or mutation of the phosphorylation acceptor Ser544 to Ala significantly increased ectodomain shedding, whereas overexpression of CK2ε inhibited cleavage of collagen XVII. Structural modeling suggested that the phosphorylation of serine residues prevents binding of TACE to its substrate. Thus, extracellular phosphorylation of collagen XVII by ecto-CK2 inhibits its shedding by TACE and represents novel mechanism to regulate adhesion and motility of epithelial cells.

Cells need a versatile, fast mechanism to respond to changes in their microenvironment during development, growth, and regeneration. Regulated proteolysis on the cell surface offers a post-translational mechanism to remove or structurally modify surface associated proteins at any time (1). For a multitude of type I and type II transmembrane proteins, such as receptors, cell adhesion molecules, and growth factors, soluble forms have been identified that have functions distinct from their membrane-bound counterparts. These are often generated through ectodomain shedding, a general mechanism that influences interactions of different cell types with the environment in a broad spectrum of biological and pathological processes (1, 2). However, regulation of the ectodomain shedding remains poorly understood, namely, targeting of the sheddase to particular substrate depending on biological context.

One prime example in this context is collagen XVII, a prototype of the novel family of collagenous transmembrane proteins (3). It is a structural component of hemidesmosomes that mediate adhesion of the epidermal keratinocytes to the underlying basement membrane in the skin (4). As a transmembrane component within the multiprotein complexes, collagen XVII constitutes an important link between intra- and extracellular structural elements. This is indirectly demonstrated by human diseases, caused by loss of collagen XVII functions. Mutations in the COL17A1 gene lead to absence or diminished expression of collagen XVII and to functional epidermolysis bullosa, a hereditary disorder characterized by dermal-epidermal separation and skin blistering (5). In bullous pemphigoid, an autoimmune blistering disease, autoantibodies targeting collagen XVII disrupt dermal-epidermal cohesion (6).

Like all collagenous transmembrane proteins, collagen XVII is positioned in the plasma membrane in type II orientation, and the ectodomain is constitutively shed from the cell surface. The cleavage occurs extracellularly, within the juxtamembrane linker domain NC16A (Fig. 1) (7). Metalloproteinases of the ADAM (a disintegrin and metalloproteinase) family catalyze this process, with ADAM17/TACE (TNF-α converting enzyme) appearing to be the major sheddase of collagen XVII (8). TACE, a transmembrane enzyme itself, is involved in the shedding of a variety of type I and II transmembrane proteins (9). Substrate recognition by TACE was thought to depend on structural motifs rather than a common consensus sequence (9). However, certain sequence preferences in recognition have...
of intracellular substrates, and it is important for the control of cell cycle progression and apoptosis (18). Intriguingly, it is also active as an ecastroprotein kinase (19–21), phosphorylating proteins such as amyloid precursor protein (22), laminin-1 (23), osteopontin (24), and vitronectin (25). This seems to link ecastro-CK2 activity to cell adhesion and migration processes, as well as to bone formation and pathogenesis of Alzheimer disease. In general, phosphorylation is a well established mechanism of modulating the interaction between individual proteins, including interactions of proteases with their substrates (26, 27). Here, we identified collagen XVII as a new extracellular substrate of ecastro-CK2 and show that serine phosphorylation within the NC16A domain interferes with ectodomain shedding, presumably by perturbing the binding of collagen XVII to TACE.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transient Transfections**—HaCat cells were cultured in keratinocyte growth medium supplemented with bovine pituitary extract and epidermal growth factor as described (28). COS-7 cells were grown in DMEM containing 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. All media and supplements were obtained from Invitrogen. Transfection of 90% confluent cells was carried out with 1 µg of DNA/0.5 × 10⁶ cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. 48 h prior to harvest, the medium was supplemented with 50 µg/ml l-ascorbate.

**Preparation of cDNA Constructs**—Full-length collagen XVII cDNA inserted into pcDNA3 was a generous gift of Dr. L. Borra- dori, University of Geneva. The plasmids encoding HA-tagged CK2α and CK2α K58A were kindly provided by Dr. Stephan Bek (Max-Planck-Institute of Immunobiology, Freiburg). The EcoRV/Clal fragment spanning nucleotides 1374–2548 of human collagen XVII cDNA was subcloned into pBluescript II SK (Stratagene) and subjected to site-directed mutagenesis with the following primers: 5'-GACAAAAATGCGCTGCAGGAGTACGGCCAGGAGGAGCTCCTC-3' and 5'-AGAGCTCCTCCTGCGGTCCACTGTGCAGGCTTATTTTGC-3' for collagen XVII by the ecastro-protein kinase casein kinase 2 (CK2) and for its role in the regulation of ectodomain shedding.

Extracellular phosphorylation is emerging as a novel mechanism in regulation of many biological processes, such as cell adhesion and proliferation, or mechanisms of immune response (15). Ecastro-protein kinases act on the outer surface of the plasma membrane and use extracellular nucleoside-triphosphates as co-substrates. They phosphorylate membrane-bound proteins, extracellular matrix proteins, and soluble factors, using extracellular ATP as a source of the phosphate group (15). The nucleotides can be derived from the intracellular pool through selective membrane permeabilization, transport in vesicles, or cell disruption (15). Alternatively, extracellular ATP can be synthesized from cAMP and ADP (15, 16).

CK2 is a highly conserved protein kinase expressed in nearly every eukaryotic tissue and cellular compartment (17, 18). It is active as a heterotrimer of two catalytic and two regulatory subunits and phosphorylates mainly serine or threonine residues, with some tyrosine kinase activity (17). CK2 has a variety...
buffer (70 mM NaCl, 5 mM (CH₂COO)₂Mg, 0.5 mM EDTA, and 5 mM KH₂PO₄/K₂HPO₄, 30 mM Tris, pH 7.3) supplemented with 330 μCi/ml [γ-³²P]ATP (GE Healthcare, Munich, Germany) in the presence or absence of 30 μM TBB or 1 μg/ml K252b. Phosphorylation was terminated by extensive washing of the cells with ice-cold phosphorylation buffer containing 2 mM unlabeled ATP. Subsequently, cells were lysed for 10 min on ice in a buffer containing 1% Nonidet P-40, 0.1 M NaCl, 25 mM Tris-HCl, pH 7.4, 10 mM EDTA, 2 mM ATP, proteinase inhibition mixture set III (Calbiochem) and phosphatase inhibition mixture 1 (Sigma-Aldrich). The lysates were clarified by centrifugation, and collagen XVII was immunoprecipitated with the collagen XVII antibody endo-2 (6). After extensive washing, the immunoprecipitated proteins were eluted with SDS sample buffer under reducing conditions at 95 °C and separated by SDS-PAGE. Gels were stained with Coomassie Blue (Bio-Rad), and radiolabeled proteins were detected by phorimaging. Expression of collagen XVII was monitored by incubation of HaCaT keratinocytes with 30 μM TBB or 1 μg/ml K252b for 60 min, followed by lysis and immunoblotting with endo-2 antibodies (6).

**CK2 in Vitro Phosphorylation Assay**—Phosphorylation of synthetic peptides corresponding to amino acid residues 530–559 of wild-type collagen XVII, AAGADLKIGLH (Sigma-Aldrich) in the presence or absence of 30 μM TBB or 1 μg/ml K252b for 60 min, followed by lysis and immunoblotting with endo-2 antibodies (6).

**Phosphoepitope Antibodies**—The antibody pSer544 was raised by immunizing rabbits with the peptide D(phos)QEELWMFVRKKLMME immobilized on streptavidin-Sepharose-4B gel (Invitrogen). After elution, the specificity of the purified antibody was determined by dot-blot analysis using 0.1 μg of phosphorylated and unphosphorylated biotinylated peptide as an antigen on an SAM2 Biotin Capture membrane (Promega, Madison, WI).

**Phosphatase Treatment**—350 μl of lysate of subconfluent HaCaT cells was supplemented either with 2 mM MnCl₂ and 800 units of 1 phosphatase (New England Biolabs) or the phosphatase inhibition mixture 1 (Sigma-Aldrich) and incubated for 30 min at room temperature. The reaction was stopped by boiling the probes for 3 min in SDS sample buffer, and the lysates were immunoblotted with pSer544, NC16A antibody (4) or anti-phospho mitogen-activated protein kinase p42/p44 antibody (Sigma-Aldrich).

**Epidermal Extracts**—The epidermis was separated from the dermis by incubation in a buffer containing 1 M NaCl, 50 mM Tris, pH 7.4, 1 μM Pefabloc, 10 mM EDTA, phosphatase inhibition mixture 1 (Sigma-Aldrich) at 4 °C for 5 h under constant stirring. The epidermis was mechanically peeled off and briefly washed with phosphate-buffered saline, and proteins were extracted by a 5-min incubation in SDS sample buffer containing 1 mM dithiothreitol at 95 °C.

**Collagen XVII Extraction and Shedding Assays**—Cell culture medium and cell extracts were collected and processed separately, as described (8). Briefly, the medium was collected on ice, supplemented with 1 mM Pefabloc (Merck) and 1 mM EDTA (Sigma) and clarified from debris by centrifugation at 200 × g for 5 min at 4 °C. The cells were washed twice with phosphate-buffered saline and lysed for 20 min in a buffer containing 1% Nonidet P-40, 0.1 M NaCl, proteinase inhibition mixture set III (Calbiochem), and 25 mM Tris-HCl, pH 7.4. The cell lysate was collected and centrifuged at 13,000 × g for 20 min at 4 °C. Cell culture supernatants were precipitated with ethanol, dissolved in SDS-PAGE sample buffer, normalized in protein content, and analyzed by immunoblotting with the NC16A antibody. Semiquantitative densitometry was performed with the Gel-Pro Express 4.0 software (Media Cybernetics Inc.).

The effect of extracellular phosphorylation on shedding was determined by treatment of HaCaT keratinocytes with 1–2 μg/ml K252b, 75–150 μM TBB, and 75 μM D4476 in keratinocyte growth medium (Calbiochem) for 120 min prior to extraction. To estimate the effect of CK2 overexpression on shedding, COS-7 cells were co-transfected with a cDNA for collagen XVII and for HA-tagged CK2α or the catalytically inactive mutant CK2α K58A and analyzed 48 h after the transfection, as described above. The presence of both CK2 variants in the extracellular space was confirmed by precipitating the culture medium with ethanol at −20 °C and immunoblotting with anti-HA tag antibodies (Sigma-Aldrich). Shedding in COS-7 cells transfected with wild-type collagen XVII and the mutants collagen XVII (S544A) or collagen XVII (S[542,544]A) was analyzed 24 h after the transfection, as described above.

**TACE Enzyme-linked Immunosorbent Assay and Activity Assay**—HaCaT keratinocytes were treated with 1–2 μg/ml K252b and 75–150 μM TBB in keratinocyte growth medium for 120 min, and expression and activity of TACE were determined in parallel. The expression levels of TACE were assayed by Quantikine human TACE/ADAM17 Immunoassay (R&D Systems) according to the manufacturer’s protocol. Briefly, the cells were lysed with the provided lysis buffer supplemented
with proteinase inhibition mixture set III (Calbiochem) and phosphatase inhibition mixture 1 (Sigma-Aldrich), and TACE concentration was determined by using a sandwich enzyme immunoassay. TACE specific activity in lysates was determined by in vitro enzymatic assay with TACE fluorogenic peptide substrate III (R&D systems). First TACE was immobilized onto a microplate coated with a monoclonal antibody against TACE (kit component 892721), and after extensive washing, 10 μM TACE substrate peptide in 25 mM Tris, pH 8.0, was added for 120 min. The TACE inhibitor TAPI-0 (TNF-α protease inhibitor 0; Calbiochem) was added as a negative control for assay specificity. Fluorescence was measured by the microplate reader Infinite 200 (Tecan Group Ltd., Germany) with excitation at 320 nm and emission at 405 nm.

Molecular Docking—Modeling of the interactions between TACE and its substrates was performed with AutoDock 3.0 (29), utilizing a Lamarckian genetic algorithm. AutoDock 3.0 uses a force field-base empirical free energy-scoring function, taking into account potential energies (Lennard-Jones dispersion/repulsion interactions, hydrogen bonds, and Coulombic electrostatic potentials) and the unfavorable entropy of ligand binding. For the zinc ion involved in the binding, we used previously described force field parameters ($R = 0.87\,\text{Å}$, $e = 0.35\,\text{kcal/mol}$, and the formal charge of $+0.95e$) (30). All available structures of TACE-ligand complexes (PDB accession numbers 1BKC, 1ZXC, 2A8H, 2DDF, and 2FV9) were used for testing of docking parameters. The active site of TACE was defined using a grid of $80 \times 80 \times 80$ points with a grid spacing of 0.375 Å centered on the ligand in the published crystal structure (31). We performed 100 independent runs with a maximum number of 27,000 Lamarckian genetic algorithm operations on a single population of 50 individuals.

The high resolution structure of TACE provided in PDB accession number 1BKC was used as a target for molecular docking of peptides representing pro-TNFα (75QAVRS79) and collagen XVII (538GLHS542). All possible torsion angles within the peptides were treated as flexible, and a water molecule was placed near the zinc ion and the side chain of Glu406. The structures of QAVRS, which showed a minimal distance between the water molecule and the hydrolyzed 76Ala-Val77 peptide bond, and the IGHLS structure corresponding to it were further refined using the energy-minimization routine of Gromacs software (32). Transition state-like structures were obtained by applying additional harmonic potential between the oxygen of the water molecule and the carbon atom of Ala (Gly) residues of QAVRS and IGHLS during energy minimization.

RESULTS

Collagen XVII Is Extracellularly Phosphorylated by CK2—Phosphorylation is known to regulate protein-protein interactions, including the binding of proteases to their substrates. Based on the preliminary findings of serine phosphorylation in collagen XVII derived from a carcinoma cell line (14), we first determined the phosphorylation status of authentic collagen XVII in HaCaT keratinocytes. To reveal potential extracellular phosphorylation of collagen XVII, we used labeling with cell-impermeable [γ-32P]ATP, a standard assay of extracellular phosphorylation (23). Because ATP cannot permeate the plasma membrane, incubation of intact cells with [γ-32P]ATP leads to selective incorporation of radioactive phosphate groups into extracellular protein domains in the presence of ecto-protein kinases. After the labeling, collagen XVII was immunoprecipitated from cell lysates with the antibody endo-2 recognizing the intracellular domain, separated by SDS-PAGE, and detected by phosphorimaging (upper panel). Equal loading of the gel was confirmed by Coomassie staining (not shown). HaCaT serine kinase II expression was not influenced by incubation of the cells with K252b or TBB, as shown by immunoblotting of the cell extracts with the antibody endo-2 (middle panel, arrow). Addition of TBB or K252b lowered the ratio of labeled collagen XVII to total collagen XVII to 28 and 52% of the control, respectively (lower panel). Bars indicate ± S.E.

![Extracellular phosphorylation of authentic collagen XVII](image-url)

**FIGURE 2.** Extracellular phosphorylation of authentic collagen XVII. HaCaT keratinocytes were incubated with cell-impermeable [γ-32P]ATP for 60 min in the presence or absence of the CK2 inhibitor TBB (30 μM) or the broad-spectrum extracellular kinase inhibitor K252b (1 μg/ml). After extensive washing, collagen XVII was immunoprecipitated from cell lysates with the antibody endo-2 recognizing the extracellular domain, separated by SDS-PAGE, and detected by phosphorimaging (upper panel). Equal loading of the gel was confirmed by Coomassie staining (not shown). HaCaT serine kinase II expression was not influenced by incubation of the cells with K252b or TBB, as shown by immunoblotting of the cell extracts with the antibody endo-2 (middle panel, arrow). Addition of TBB or K252b lowered the ratio of labeled collagen XVII to total collagen XVII to 28 and 52% of the control, respectively (lower panel). Bars indicate ± S.E.
protein kinase p42/p44 antibodies were employed. With affinity-purified pSer544, but not with the NC16A antibody. As a control, anti-phospho mitogen-activated protein kinase treatment.

Casein kinase 2 phosphorylates Ser544 and Ser542 in collagen XVII. A, synthetic peptides corresponding to amino acids 530–559 in normal collagen XVII (WT), mutated collagen XVII (Ser544 → Ala) or doubly mutated collagen XVII (Ser[542,544]Ala) were used for in vitro phosphorylation with CK2 in the presence of [γ-32P]ATP. Phosphorylation of the peptides was quantified as described under “Experimental Procedures.” Note that the mutations abolished the phosphorylation. B, the antibody pSer544 specifically recognizes the phosphorylated epitope in dot blots with the peptides AAGADLDKIGLH and AAGADLDKIGLHSD(phos)QEELWMFVRKLMME (+P) and AAGADLDKIGLHSDQEEELWMFVRKLMME (−P). The control antibody NC16A recognizes both peptides similarly. C, λ phosphatase treatment of HaCaT keratinocyte extracts abolished the reactivity of collagen XVII with affinity-purified pSer544, but not with the NC16A antibody. As a control, anti-phospho mitogen-activated protein kinase p42/p44 antibodies were employed. +P, before λ phosphatase treatment; −P, after λ phosphatase treatment. D, immunoblot of a human epidermis extract with pSer544 antibodies applied in dilutions of 1:200 to 1:10,000, as a control, the NC16A antibody was used.

Table 1

The antibodies pSer544 are specific for the phosphoepitope

For analysis of pSer544 antibody specificity, biotinylated peptides representing the phosphorylated and unphosphorylated epitopes of collagen XVII were bound to streptavidin-coated plates and enzyme-linked immunosorbent assay was performed with pSer544 antibodies applied in dilutions of 1:200 to 1:10,000, as a control, the NC16A antibody was used.

| Antibody/dilution | Non-phospho | Phos (Ser544) |
|-------------------|-------------|--------------|
| pSer544/1:200     | 0.011 ± 0.003 | 0.347 ± 0.024 |
| pSer544/1:500     | 0 ± 0.004    | 0.334 ± 0.015 |
| pSer544/1:1,000   | 0 ± 0.002    | 0.315 ± 0.014 |
| pSer544/1:2,000   | 0 ± 0.016    | 0.236 ± 0.038 |
| pSer544/1:10,000  | 0 ± 0.002    | 0.116 ± 0.015 |
| NC16A poly/1:500  | 0.393 ± 0.013 | 0.440 ± 0.064 |

TBB (4,5,6,7-tetramethylbenzimidazole) (35) significantly inhibited phosphorylation but did not influence the total amount of collagen XVII in the samples (Fig. 2). These observations demonstrated that the extracellular domain of collagen XVII is phosphorylated and that extracellular CK2 is involved in the process.
Inhibition of Extracellular Phosphorylation Increases Collagen XVII Ectodomain Shedding—The effect of extracellular phosphorylation on shedding of collagen XVII was evaluated by quantifying the release of the ectodomain into cell culture medium. Addition of increasing concentrations of the extracellular kinase inhibitor K252b (33) resulted in a dose-dependent increase of shedding (Fig. 4A). Similarly, treatment of the cultures with the specific CK2 inhibitor TBB (35) stimulated shedding of collagen XVII (Fig. 4B). In both cases, the stimulation of shedding was ~2-fold. As a control, the specific casein kinase 1 (CK1) inhibitor D4476 (37) was used to exclude involvement of CK1-like activity in the regulation of shedding. In contrast to K252b and TBB, addition of D4476 had no effect on shedding of collagen XVII (Fig. 4C). Thus, these observations indicate that extracellular phosphorylation of collagen XVII negatively regulates shedding of the ectodomain.

As a control, we compared expression levels and activity of TACE, because kinase inhibitors could increase TACE expression or activate the enzyme post-translationally. HaCaT cells were treated for 2 h with increasing concentrations of K252b or TBB, and expression levels and activity of TACE were measured in parallel. No increase in TACE expression was observed (Fig. 4D). The specific activity of TACE was measured using an in vitro enzyme assay with a TACE-specific fluorogenic peptide as a substrate (Fig. 4F). No significant variations in TACE activity were found. These findings exclude both increased expression and post-translational activation of TACE as causes for the enhanced shedding of collagen XVII by kinase inhibitors.

Mutations in CK2 or Collagen XVII Influence Shedding—To further substantiate the evidence linking CK2 activity and collagen XVII ectodomain shedding, COS-7 cells were co-transfected with expression constructs encoding collagen XVII and the catalytic subunit of CK2, CK2α, or its inactive mutant CK2α K58A, fused to an HA-tag. Both CK2 variants were expressed and secreted into the extracellular space, as shown by immunoblotting of the cell culture medium with an anti-HA tag antibody (Fig. 5B). Immunoblotting of collagen XVII ectodomain revealed that co-transfection of CK2α strongly reduced shedding, to ~25%, whereas co-transfection with the kinase dead mutant CK2α K58A did not have a significant effect (Fig. 5A), despite expression of both proteins at similar levels. These data implicated a direct link between ecto-CK2 mediated phosphorylation of the NC16A domain and collagen XVII ectodomain shedding. If this prediction is valid, mutation of Ser542 and Ser544 should result in increased shedding. Therefore, wild-type and mutated collagen XVII were expressed in COS-7 cells, and shedding of the ectodomain was quantified. Substitution of Ser544 by Ala, which causes a strong reduction in phosphorylation of the peptides (Fig. 3), led to an ~5-fold increase in shedding as compared with the wild-type protein (Fig. 5C). Additional substitution of Ser542 by Ala, surprisingly, abolished the effect of the Ser544 → Ala mutation and reduced shedding of the collagen XVII ectodomain to wild-type levels. A possible explanation for this unexpected observation is that substitution of Ser542 by Ala interferes with binding of TACE to collagen XVI. This possibility was tested by modeling the protein-protein interactions.

Modeling of the Interaction between TACE and the Amino Acids 538IGLHS542 of Collagen XVII—The precise peptide bond cleaved by TACE within the NC16A domain of collagen XVII is unknown. However, TACE has certain sequence preferences for recognition, i.e., lipophilic amino acids, such as leucine and valine, in the P1’ position and basic amino acids in the P2’ position (10). Our modeling is based on previous data pointing to the stretch of amino acid residues 528–547 in collagen XVII.
as crucial for sheddase recognition and cleavage (7). This stretch has only one good consensus sequence for TACE recognition, $^{539}$GLHS$^{542}$, with cleavage occurring after Gly$^{539}$, Leu$^{540}$ occupying position P1', and His$^{541}$ position P2' (Fig. 6A). To test this hypothesis, we utilized the crystal structure of TACE and investigated the binding of the IGLHS peptide to the active site by molecular docking, using AutoDock (29). AutoDock is a program previously shown to be most accurate and efficient in molecular docking of ligands into active sites of zinc metalloproteases (38), and the modeling was performed with the previously optimized parameters for zinc metalloproteinases (30). As a starting point of the analysis, a high resolution structure of a TACE-inhibitor complex was used to dock a pro-TNF$\alpha$ peptide (amino acid motif 75–79, QAVRS) into the active site of the enzyme, assuming that the structure of TACE-substrate complexes resembles the structure of the complex of TACE and peptide-like TACE-inhibitors (PDB accession number 1BKC). To achieve this docking, a water molecule was coordinated to the zinc ion in the catalytic center of TACE. After energy minimization of this structure, it was used as a template to model the interaction of the collagen XVII peptide IGLHS with the active site of TACE.

As shown in Fig. 6, it was possible to model this interaction with residue Leu$^{540}$ (position P1') filling the S1' hydrophobic cavity, His$^{541}$ in position P2' coordinated by the carbonyl and hydroxyl groups at the upper rim of the active site, and Ser$^{542}$ interacting with the polar entrance to the S3' pocket of the active site (Fig. 6B). The results obtained for collagen XVII strongly correlate with the results obtained for TNF-\(\alpha\) (supplemental Fig. S1). This structure may represent a model for the interaction of TACE and collagen XVII, with collagen XVII amino acids 539–542 binding to the catalytic center of TACE. Ser$^{542}$ seems to be directly involved in this binding, as already suggested by the minimal ectodomain shedding of the collagen XVII (S[542,544]) mutant.

**DISCUSSION**

Phosphorylation is a post-translational event with a profound impact on the biological activity and biochemical properties of a protein. Beside ample data describing intracellular phosphorylation events, increasing evidence exists for extracellular phosphorylation mediated by ectokinases and utilizing extracellular ATP or GTP (15). Extracellular nucleotides, such as ATP, are now recognized as important autocrine and paracrine factors involved in the regulation of many cellular processes in a wide range of tissues. Several postulated mechanisms exist for ATP release, involving ATP-binding cassette proteins (39), exocytotic vesicles (40), and cytolysis (41). Furthermore, nucleotides can undergo complex interconversions in the extracellular space. Extracellular nucleotides act via activation of receptors of the P2 family and as phosphate donors for ecto-protein kinases. It has been demonstrated that human keratinocytes release ATPs and utilize different mechanisms for nucleotide interconversion at the cell surface (16). Moreover, extracellular ATP was shown to inhibit the terminal differentiation of keratinocytes (42).

Activity of ectokinases has been implicated in the regulation of cell-cell and cell-ligand interactions, including adhesion of...
cells to the extracellular matrix (15). In addition to phosphorylating a variety of intracellular substrates, CK2 is involved as an ecto-CK2 (20) in extracellular phosphorylation processes (19). For example, ecto-CK2 activity regulates vascular smooth muscle cell adhesion via vitronectin phosphorylation (25) or monocyte migration through laminin-1 phosphorylation (23). Furthermore, ecto-CK2 may be involved in ossification by directly influencing mineral formation through phosphorylation of osteopontin (24).

Serine phosphorylation of collagen XVII in carcinoma cells was first observed by Kitajima et al. (14), but the finding was not pursued further. Here we determined that phosphorylation of authentic collagen XVII occurs extracellularly, because \( \gamma \)-\( \text{32P} \)ATP labeling took place under conditions that exclude participation of intracellular kinases. The phosphorylation was significantly decreased by both the broad spectrum extracellular kinase inhibitor K252b and the selective CK2 inhibitor TBB, demonstrating that extracellular phosphorylation of collagen XVII is mediated by ecto-CK2.

Phosphorylation by CK2 requires multiple acidic residues mainly downstream of the amino acid to be phosphorylated. Glutamic or aspartic acid is found in position \( n + 3 \) in the majority of known CK2 substrates and seems to be crucial for kinase function (18). Comparison of the putative ecto-CK2 phosphorylation site within the NC16A domain, 541-\( \text{HSDSQEE} \)547, with other CK2 substrates pointed to Ser544 as the primary target for phosphorylation (Fig. 1). In vitro phosphorylation of synthetic peptides by recombinant CK2 proved that 541-\( \text{HSDSQEE} \)547 is indeed recognized by the enzyme, and that both Ser544 and Ser542 can be targeted. The amino acid stretch surrounding Ser542 is less favorable for recognition by CK2, because position \( n + 3 \) is neither Glu nor Asp. Interestingly, however, phosphorylation of Ser544 leads to acidification of the environment for Ser542, thus perhaps allowing CK2 to phosphorylate this residue. We predict that Ser544 is the primary extracellular residue to be phosphorylated in collagen XVII and that its phosphorylation allows the recognition of Ser542 by CK2, leading to sequential phosphorylation of both residues. After showing that synthetic peptides of collagen XVII can be phosphorylated by CK2 in vitro, we generated an antibody against the primary phosphoepitope. The antibody specifically recognized the phosphorylated form of collagen XVII in extracts of HaCaT keratinocytes and of human epidermis, proving that phosphorylation of Ser544 is a naturally occurring event in the skin.

Phosphorylation is known to regulate susceptibility of individual proteins to proteases. For example, CK2-mediated phosphorylation antagonizes degradation of proteins by caspases. The proteins Max (43), Bid (44), and connexin (45) become inaccessible to caspase cleavage upon phosphorylation by CK2. In these cases, the phosphorylated residue was very close to the caspase cleavage site, suggesting that the adverse effect of CK2 was site-associated. Here, we used kinase inhibitors to show that extracellular phosphorylation of collagen XVII negatively regulates its shedding by TACE. Moreover, overexpression of CK2\( \alpha \) inhibited shedding, in contrast to an inactive CK2\( \alpha \) mutant, CK2\( \alpha \)K58A. Accordingly, substitution of Ser544 by Ala, which reduced phosphorylation of collagen XVII, strongly enhanced the shedding, indicating that ecto-CK2 phosphorylation of Ser544 attenuates the cleavage of collagen XVII ectodomain by TACE.

TACE, a transmembrane zinc-endopeptidase, is involved in shedding of a variety of type I and II transmembrane proteins (9). It is synthesized as a latent pro-enzyme that requires post-translational processing for activation. The pro-domain is cleaved by furin, which removes an inhibitory peptide from the active site of TACE. This peptide contains a cysteine residue that coordinates with and masks the zinc of the catalytic domain (Fig. 6A). The crystal structure of the catalytic domain of human TACE (31) provides valuable insight into the mechanism of substrate binding to the active site. Based on this structure, a model was proposed for pro-TNF\( \alpha \) binding to TACE, with the isopropanyl side chain of Val77 (P1') fitting into the

![Image](50x336 to 299x734)

**FIGURE 6.** Model for the interaction of TACE and collagen XVII. **A**, a schematic representation of the structural preferences for TACE binding. As shown for the TACE-inhibitory peptide, pro-TNF\( \alpha \), and Notch, TACE prefers the lipophilic residues Val and Leu at position P1' and preferentially binds basic amino acid residues at position P2'. Note the predicted TACE binding site of collagen XVII, with the horizontal line indicating the position of cleavage. **B**, representation of the structure of the collagen XVII/TACE interface, as obtained by molecular docking of the 538-IGLHS542 sequence of collagen XVII to the TACE active site. The carbohydrate chain is the 538-IGLHS542 sequence, the red globe depicts a zinc ion, and the pink globe represents a water molecule. The molecule surfaces are colored according to charge: negatively charged areas in red, positively charged areas in blue.
hydrophobic S1’ neck, Arg78 (P2’) anchoring to the carbonyl and hydroxyl groups at the upper rim of the S2’, and the hydroxyl group of Ser79 interacting with the polar entrance of the S3’ pocket. The preceding amino acid stretch 74AEA76 of pro-TNFα aligns to the bound strand in an antiparallel manner, forming a loop. The residues downstream of the cysteine in the TACE-inhibitory propeptide, Val and Lys (46), are probably bound to the active site of TACE in a manner similar to the pro-TNFα Val77–Arg78 sequence.

Collagen XVII contains the 540LHS542 sequence motif, with clear functional similarity to 77VRS79 of pro-TNFα. This motif is located within amino acid sequence 528–547, which was previously shown to be important for recognition and cleavage of the ectodomain by TACE (7). In the present study we provide additional evidence for the importance of the 540LHS542 region using the collagen XVII mutants. Although substitution of Ser544 with Ala enhanced shedding due to loss of inhibitory phosphorylation, substitution of both Ser544 and Ser542 with Ala resulted in minimal release of the ectodomain from the cell surface. This suggested a direct involvement of Ser542 in binding of collagen XVII to TACE. By molecular docking we showed that the motif 538IGLHS542 in collagen XVII can fit into the active site of the enzyme. The structure obtained for the complex from the furin site105RRRR108 (49). It is tempting to speculate that ectodomain shedding of collagens XIII and XXIII and ectodysplasin A is also regulated by extracellular phosphorylation, which antagonizes the action of the sheddases.

The functional consequences of ectodomain shedding of collagen XVII comprise regulation of epithelial cell adhesion, detachment and motility during development, differentiation and regeneration, as well as tumorigenesis. Other collagenous transmembrane proteins engage in a broad spectrum of biological functions, ranging from epithelial morphogenesis to neuromuscular signaling and immunological host defense (3). Consequently, extracellular phosphorylation of transmembrane proteins is likely to represent a general regulatory mechanism in different cells and tissues. It is well documented that ecto-protein kinases on the outer cell surface can phosphorylate both extracellular substrates and cell surface proteins (15). Their localization and substrate specificity suggest that they influence cell-cell interactions, ligand binding, and signal transduction and, through these mechanisms, a multitude of cellular functions under physiological and pathological conditions.

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