Identification of Novel Interaction between ADAM17 (a Disintegrin and Metalloprotease 17) and Thioredoxin-1∗

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Background: The identification of potential interaction partners for TACE could be instrumental in understanding the regulation of TACE activity.

Results: Trx-1 interacts with the cytoplasmic domain of ADAM17.

Conclusion: Trx-1 regulates ADAM17 activity.

Significance: The data suggest a negative ADAM17 regulation in the HB-EGF shedding model.

ADAM17, which is also known as TNFα-converting enzyme, is the major sheddase for the EGF receptor ligands and is considered to be one of the main proteases responsible for the ectodomain shedding of surface proteins. How a membrane-anchored protease with an extracellular catalytic domain can be activated by inside-out regulation is not completely understood. We characterized thioredoxin-1 (Trx-1) as a partner of the ADAM17 cytoplasmic domain that could be involved in the regulation of ADAM17 activity. We induced the overexpression of the ADAM17 cytoplasmic domain in HEK293 cells, and ligands able to bind this domain were identified by MS after protein immunoprecipitation. Trx-1 was also validated as a ligand of the ADAM17 cytoplasmic domain and full-length ADAM17 recombinant proteins by immunoblotting, immunolocalization, and solid phase binding assay. In addition, using nuclear magnetic resonance, it was shown in vitro that the titration of the ADAM17 cytoplasmic domain promotes changes in the conformation of Trx-1. The MS analysis of the cross-linked complexes showed cross-linking between the two proteins by lysine residues. To further evaluate the functional role of Trx-1, we used a heparin-binding EGF shedding cell model and observed that the overexpression of Trx-1 in HEK293 cells could decrease the activity of ADAM17, activated by either phorbol 12-myristate 13-acetate or EGF. This study identifies Trx-1 as a novel interaction partner of the ADAM17 cytoplasmic domain and suggests that Trx-1 is a potential candidate that could be involved in ADAM17 activity regulation.

ADAMs (a disintegrin and metalloproteinase) comprise a family of membrane-associated metalloproteinases with a complex multidomain structure composed of the following domains: metalloproteinase, disintegrin-like, cysteine-rich, epidermal growth factor-like, transmembrane, and cytoplasmic domains. ADAM17, also known as the TNFα-converting enzyme, is responsible for cleaving of several growth factors, cytokines, and cell surface receptors (1).

Progress has been made to identify the signaling molecules that participate in the induction of ADAM17 shedding. Several reports have questioned whether the cytoplasmic domain is involved (2) or not involved (3) in ADAM17 activation, and some mechanisms have been proposed, such as the following: 1) phosphorylation of its cytoplasmic domain at Thr735 by p38 MAP kinase (2); 2) EGF receptor activation by G-protein-coupled receptors involving the Src-dependent phosphorylation of ADAM17 (4); and 3) Erk MAP kinase signaling (5, 6). However, Le Gall et al. (3) showed that ADAM17 activity does not depend on intracellular signaling through the ADAM17 cytoplasmic tail, suggesting that the regulation may occur through the transmembrane domain. Additionally, it has been shown that the down-regulation of thiol isomerases enhanced ADAM17 activity by inducing changes in the redox environment, and thus, a protein disulfide isomerase could be a specific regulator (7). In this context, Zhang et al. (8) suggested that H2O2 can activate ADAM17 through oxidative attack of a pro-domain thiol group and therefore lead to the disruption of its inhibitory coordination with the Zn2+ in the catalytic domain. In addition, many authors have shown the involvement of reactive oxygen species (ROS) in ADAM17 activation by p38 MAP kinase (9, 10).

Several proteins have been identified as ADAM17 cytoplasmic domain interaction partners, such as MAD2 (11), PTPH1 (12), Erk1/2 (6), FHL2 (13), and p38α MAPK (2). The cytoplasmic domain of ADAM12 has also been described as a partner of c-Src/Yes (14, 15), Grb2 (15), PI3K (16), α-actinin-1 (17), Tks5/Fisk (18), PACSIN3 (19), Eve-1 (20), and PKCe (21). However, most of these partners are not necessarily related to the proteolytic activation.

The data suggest a negative ADAM17 regulation in the HB-EGF shedding model.
Thioredoxin-1 Is a Novel Interaction Partner of ADAM17

TABLE 1

Vectors, cloned residues, and primer sequences used for ADAM17 cytoplasmic domain and Trx-1 constructions

| Construction                  | Residues | Vector          | Primer sequences                                                                 |
|-------------------------------|----------|----------------|----------------------------------------------------------------------------------|
| FLAG-ADAM17cyto               | 693–824  | pcDNA3         | 5'-AAGAATCATGGACATCAAAGACGATGACGAACG' (EcoRI)                                     |
| His-FLAG-ADAM17cyto           | 693–824  | pET 28a (+)    | 5'-AATCTAGATTAGCACTCTGTTCTTTCGC-3' (XbaI)                                        |
| Trx-1-FA                      | 1–105    | pcDNA3         | 5'-TTATCTAGATTAGCACTCTGTTCTTTCGC-3' (NheI)                                        |
| His-Trx-1-FA                  | 1–105    | pET-28a (+)*   | 5'-ATATGGAATCTTACGACCTCTGTTCTTTCGC-3' (BamHI)                                    |

* Subcloned from pcDNA3 Trx-1-FA between BamHI and NotI.

To correlate the activation of ADAM17 by inside-out regulation, we used multiple strategies to identify ADAM17 cytoplasmic domain partners and cell-based assays to analyze the functional role of the partner in ADAM17 activation. In the present study, we have demonstrated the following: 1) Trx-1 is an ADAM17 cytoplasmic domain ligand in HEK293 cells; 2) Trx-1 co-localized with the ADAM17 cytoplasmic domain and full-length ADAM17 recombinant proteins; 3) Trx-1 directly interacts with the ADAM17 cytoplasmic domain; 4) the overexpression of Trx-1 recombinant protein in the presence of PMA, which generates reactive oxygen species (ROS), decreases ADAM17 activity; and 5) the physiological shedding of ADAM17 substrate, such as EGF, is modulated by interaction with Trx-1. These results suggest that Trx-1 is a direct interaction partner of the ADAM17 cytoplasmic domain and could be involved in the modulation of ADAM17 activity.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293 (human embryonic kidney), HeLa (cervical cancer cells), and SV40-transformed mEF cells (mouse embryonic fibroblast) were cultured in DMEM with 10% FBS (cervical cancer cells), and SV40-transformed mEF cells (mouse embryonic fibroblast) were cultured in DMEM with 10% FBS (cervical cancer cells), and SV40-transformed mEF cells (mouse embryonic fibroblast) were cultured in DMEM with 10% FBS. The WT mEF cells and Adami7–/– mEF cells were kindly provided by Dr. Carl Blobel (Weill Medical College of Cornell University) (22).

Constructions—Human ADAM17 cytoplasmic domain and human Trx-1 were cloned from a cDNA library, generated from HEK293 cells. Vectors, primers sequences, insert restriction sites, and cloned residues are described in the Table 1. Full-length HA-tagged ADAM17 was kindly supplied by Dr. Axel Ullrich (Department of Molecular Biology, Max-Planck Institute of Biochemistry) (23). Plasmid encoding HB-EGF-AP, a chimeric protein used for alkaline phosphatase (AP) reporter assay, was kindly provided by Dr. Michael R. Freeman (Department of Surgery, Harvard Medical School, Boston) (24–26).

The negative control for immunoprecipitation (IP) experiments was a FLAG-tagged GFP, named FLAG-GFP, cloned into a pcDNA3 vector (Invitrogen) or the empty pcDNA3.

Transient Transfections—HEK293 and HeLa cells were transiently transfected with vectors encoding FLAG-ADAM17cyto, ADAM17-FA, and FLAG-GFP using the Lipofectamine and Plus reagent (Invitrogen), following manufacturer’s instructions. Protein overexpression was performed for 48 h for IP and confocal co-localization experiments. Trx-1-FA was also co-transfected in HEK293 cells stable expressing HB-EGF-AP using polyethyleneimine (Polysciences Inc.) for 48 h. WT and Adam17+/– mEF cells were transiently transfected with vector encoding HB-EGF-AP as described before.

Stable Transfections—For alkaline phosphatase reporter assay, HEK293 cells were transfected using polyethyleneimine (Polysciences Inc.), and the stable transfected cells (mix population) were selected by G418 (Invitrogen) at 500 μg/ml for at least 2 weeks.

Immunoprecipitation Experiments—To identify ADAM17 cytoplasmic binding partners, HEK293 cells were seeded in five 150-mm dishes (Corning) until 50–60% confluence and after transient transfections with FLAG-ADAM17cyto, the cells were washed with PBS, centrifuged for 5 min at 150 × g and resuspended in 10 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with phosphate inhibitors (1 mM Na2VO4, 10 mM Na3O2P2, 10H2O, 1 mM NaF, 1 mM C3H2Na2O3P2H2O; Sigma). After 30 min of incubation on ice, the cell lysates were centrifuged for 10 min at 10,000 × g at 4 °C to clear cell debris. The cell lysates were incubated at 4 °C overnight under gentle agitation with 150 μl of anti-FLAG M2 affinity gel (Sigma). The beads were washed three times with TBS, and the immunocomplexes were eluted with FLAG peptide (Sigma) at a final concentration of 150 ng/μl during 2 h at 4 °C, under agitation. The experiment was performed three times, and the samples were analyzed by LC-MS/MS.

To confirm the interaction between Trx-1 and full-length ADAM17-FA, HEK293 cells were transfected and lysed using the following lysis buffer (250 mM NaCl, 50 mM Tris, pH 8.0, containing 5 mM EDTA, 0.5% Igepal, and protease inhibitors (complete mini-EDTA free; Roche Applied Science). For the resin preparation, 100 μl of protein G-Sepharose (GE Healthcare) and 2 μg of anti-HA antibody were added to 1 ml of lysis buffer and incubated for 1 h at room temperature under gentle agitation. The resin was spun down at 12,000 × g, and 300 μg of protein extract was added to the pellet and incubated for 2 h at 4 °C under gentle agitation. The resin was then washed six times with lysis buffer, and the proteins were eluted by adding Laemmli sample buffer. The interaction of ADAM-17-FA and endogenous Trx-1 were analyzed by immunoblotting.

To evaluate whether the endogenous Trx-1 interaction is dependent on independent of phosphorylation, HEK293 cells were transiently transfected with FLAG-ADAM17cyto as described above. After 48 h, the cells were washed three times with PBS and treated with PMA (50 ng/ml) or Me3SO for 1 h.
The proteins were immunoprecipitated and analyzed by LC-MS/MS. The data analyses were performed using QualBrowser software (Thermo Xcalibur version 2.1), which calculates the area under the curve of extracted ion chromatogram (XIC) of peptides in a narrow m/z range corresponding to peptides from potential interaction partners and/or phosphorylation sites.

Analysis of ADAM17 Cytoplasmic Domain Partners by LC-MS/MS—The immunocomplexes were reduced (5 mM dithiothreitol, 25 min at 56 °C), alkylated (14 mM iodoacetamide, 30 min at room temperature in the dark), and digested with trypsin (Promega). The samples were dried in a vacuum concentrator and reconstituted in 20 μl of 0.1% formic acid. 4.5 μl of the resulting peptide mixture was analyzed on an ETD enabled LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with LC-MS/MS by an EASY-nLC system (Proxeon Biosystem) through a Proxeon nanoelectrospray ion source. Peptides were separated by a 2–90% acetonitrile gradient in 0.1% formic acid using a pre-column EASY-Column (2 cm × inner diameter of 100 μm, 5-μm particle size) and an analytical column PicoFrit Column (20 cm × ID75 μm, 5-μm particle size; New Objective) at a flow rate of 300 nl/min over 45 min. The nanoelectrospray voltage was set to 2.5 kV, and the source temperature was 200 °C. All of the instrument methods for the LTQ Velos Orbitrap were set up in the data-dependent acquisition mode. The full scan MS spectra (m/z 300–2,000) were acquired in the Orbitrap analyzer after accumulation to a target value of 1 e6. Resolution in the Orbitrap was set to r = 60,000, and the 20 most intense peptide ions with charge states ≥2 were sequentially isolated to a target value of 5,000 and fragmented in the linear ion trap by low energy CID (normalized collision energy of 35%). The signal threshold for triggering an MS/MS event was set to 1,000 counts. Dynamic exclusion was enabled with an exclusion size list of 500, an exclusion duration of 60 s, and a repeat count of 1. An activation of q = 0.25 and an activation time of 10 ms were used.

Peak lists (msf) were generated from the raw data files using Proteome Discoverer version 1.3 (Thermo Fisher Scientific) with Sequest search engine and searched against Human International Protein Database, version 3.86 (91,522 sequences; 36,630,302 residues, release July 2011) with carbamidomethylation (+57.021 Da) as fixed modification; oxidation of methionine (+15.995 Da); phosphorylation of serine, threonine, and tyrosine (+79.966 Da) as variable modifications; one trypsin missed cleavage and a tolerance of 10 ppm for precursor and 1.8, 2.2, and 2.5, and +4 > 3.5). Potential phosphorylation sites passing the scoring thresholds were manually validated.

Immunoblotting—For detection of ADAM17 recombinant protein and to validate partners from immunocomplexes, samples (20 μl) were heated to 95 °C for 5 min and separated by 15% SDS-PAGE. Proteins were transferred onto nitrocellulose membrane (GE Healthcare) by a semidy system (Bio-Rad). The nitrocellulose membrane was blocked with 5% skim milk for 2 h and incubated with anti-FLAG (1:5000; Sigma), anti-HA (1:2000; Sigma), and anti-Trx-1 (1:1000; Abfrontier) specific antibodies for 2 h. The membranes were washed three times, each for 5 min, with 10 ml of Tris-buffered saline containing 0.05% Tween 20 and then reacted to horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnology) or mouse anti-goat IgG (1:5000, Santa Cruz Biotechnology) for 2 h. After three washes, as described above, the visualization of ADAM17 recombinant protein and Trx-1 were achieved by chemiluminescence with the ECL kit (Amersham Biosciences).

Enrichment of Membrane Proteins—To access the expression of ADAM17 zymogen (pro-ADAM17) and its mature form, we performed the membrane protein enrichment, as described by Butler et al. (27), in HEK293 cells after Trx-1-HA overexpression followed by PMA treatment. The membrane proteins were separated by a 10% SDS-PAGE and then were transferred to nitrocellulose membrane and incubated with rabbit anti-TNFα-converting enzyme (1:2000; Millipore) antibody as described before.

Confocal Microscopy—HeLa cells were cultivated in 24-well plates containing 13-mm microscope cover glasses. After transient transfections with FLAG-ADAM17cyto, ADAM17-HA, and Trx-1-HA, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, washed again, and permeabilized with 0.5% Triton X-100 for 10 min. The cells were blocked with blocking solution (PBS containing 0.2% Triton and 3% nonfat dry milk) for 30 min and then incubated with primary antibodies diluted in blocking solution for 1 h. Anti-FLAG antibody (Sigma) was diluted to 1:200, anti-HA (Sigma) was diluted to 1:100, and anti-Trx-1 (Ab Frontier) was diluted to 1:100. The cells were washed with PBS and incubated with Alexa Fluor 488- or 568-conjugated antibodies (Invitrogen) diluted in blocking solution for 1 h. Finally, the cells were washed with PBS, incubated with DAPI solution for 10 min, washed again, and analyzed in a confocal microscope (Zeiss; LSM510).

Expression and Purification of Recombinant His-tagged Proteins—His-Trx-1-HA protein was expressed in BL21 (DE3) cells at 37 °C for 4 h after induction with 0.5 mM IPTG in LB or M9 medium supplemented with 1 g/liter [15N]ammonium chloride (Cambridge Isotopes, Inc.) (28) for cross-linking and NMR experiments, respectively. The harvested cells were resuspended in lysis buffer (20 mM sodium phosphate, pH 6.5, containing 80 mM NaCl and 1 mM PMSF) and disrupted by lysozyme treatment (100 μg/ml for 30 min on ice), followed by sonication (Vibracell VCX 500; Sonics & Materials, Inc.). The suspensions were centrifuged at 20,000 × g for 10 min at 4 °C. The supernatant was loaded onto nickel-charged 5-ml His Trap Chelating columns (GE Healthcare) using a flow rate of 1 ml/min in buffer A (20 mM sodium phosphate, pH 6.5, containing 80 mM NaCl). Proteins were eluted using a linear gradient of 0–1 M imidazole. Affinity chromatography fractions containing His-Trx-1-HA recombinant protein were concentrated using a 10-kDa cutoff Amicon filter (Millipore) and submitted to size exclusion chromatography using a Superdex 200 16/60 column (GE Healthcare) and a flow rate of 1 ml/min in buffer A. All of the chromatographic steps were performed using an AKTA FPLC system (GE Healthcare). The purified fractions were separated by 12% SDS-PAGE under denaturing conditions. Final protein concentration was determined by a BCA protein assay kit (Thermo Fisher Scientific Inc.).
His-FLAG-ADAM17cyto protein was expressed in BL21 (DE3) cells at 37 °C for 4 h after induction with 0.5 mM IPTG in LB medium. The cells were resuspended and lyzed using the same protocol used for His-Trx-1-HA expression. His-FLAG-ADAM17cyto protein purification was the same as used for the purification of His-Trx-1-HA, except that one intermediate step in a 1-ml MonoQ ion exchange column (GE Healthcare) was necessary between the affinity and size exclusion chromatography. This ion exchange chromatography was carried out using a flow rate of 1 ml/min in buffer A (20 mM Tris-HCl, pH 8.8, containing 20 mM NaCl). The proteins were eluted using a linear gradient of 0–1 M NaCl. The protein concentration was determined as described before.

**Solid Phase Binding Assay**—One μg of purified His-FLAG-ADAM17cyto or His-Trx-1-HA were immobilized into a 96-well polystyrene High Bind microtiter plate (Corning Glass) in a 0.05 M sodium carbonate buffer, pH 9.6, with gentle agitation overnight at 4 °C, as described by Oliveira et al. (29). The wells were washed three times with wash solution (PBS supplemented with 0.05% Tween 20) and then blocked with PBS containing 5% nonfat dry milk for 2 h at room temperature. After blocking, the wells were washed again and the purified proteins were added in increasing concentration (0.5–16 nM), diluted in PBS supplemented with 0.05% Triton X-100. The plates were incubated for 2 h at room temperature, after which the wells were washed three times. We detected the protein binding using a colorimetric ELISA. We incubated the complexes for 1 h at room temperature with anti-Trx-1 (Ab Frontier) or anti-FLAG (Sigma) antibodies, both diluted 1:1000. The wells were washed three times and incubated with a peroxidase-linked horse anti-rabbit secondary antibody (Calbiochem) diluted 1:10000 for 1 h at room temperature. After incubation with secondary antibody, the wells were washed three times and developed with a buffer containing 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma), 0.1 M citric acid, and 0.03% H2O2 (Merck). The absorbance was measured at 405 nm. The experiment were performed in triplicate and repeated twice.

**Nuclear Magnetic Resonance Spectroscopy Analysis**—Experiments that correlate the frequencies of the amide proton and the nitrogen for each amino acid in the protein can yield information about interaction sites in the protein, as well as the strength of the interaction (30). The signals that change positions in the 15N-HSQC spectra of the Trx1 protein correspond to main chain amide groups from amino acids that interacted with the unlabeled ADAM17cyto protein. NMR experiments were performed using a Varian/Agilent Inova spectrometer operating at a 1H Larmor frequency of 599.887 MHz and temperature of 25 °C. For these experiments, the spectrometer was equipped with a triple resonance cryogenic probe and a Z pulse-field gradient unit. The 15N-labeled Trx-1 sample was dissolved in 20 mM phosphate buffer, pH 6.5, containing 80 mM NaCl and 5% (v/v) D2O, at a final concentration of 0.7 mM. Water suppression was achieved by low power continuous wave irradiation over the relaxation delay or using the WATERGATE method. All of the data were processed using NMRPipe and NMRVIEW software packages (31, 32). Prior to Fourier transformation, the time domain data were zero-filled in all dimensions. When necessary, a fifth order polynomial base-line correction was applied after transformation and phasing. Experiments were performed in one and two dimensions, with double resonance techniques to correlate 1H and 15N from the main chain amino acids (15N-HSQC) (33). The hydrogen proton one-dimensional spectrum was acquired to verify that the protein was structured, and two-dimensional experiments (15N-HSQC) (32) were performed to identify interactions between the proteins His-Trx-1-HA and His-FLAG-ADAM17cyto. For this, a His-Trx-1-HA protein sample was labeled with 15N and the His-FLAG-ADAM17cyto protein expressed in unlabeled media (LB) and titrated in different proportions (50:1, 25:1, 10:1, 5:1, 1:1, and 2:1, respectively).

**Cross-linking Analysis between His-FLAG-ADAM17cyto and His-Trx-1-HA by Mass Spectrometry**—Cross-linking reactions were performed by incubating the 5 × 10–10 mol of each purified cytoplasmic domain and purified Trx-1 recombinant proteins with 1.25 mM disuccinimidyl suberate (DSS, spacer arm length: 11.4 Å, Sigma-Aldrich) for 2 h at room temperature, followed by quenching with Laemmli sample buffer. DSS-cross-linked cytoplasmic domain-Trx-1 complexes were identified as shifted bands in 12% SDS-PAGE. The protein complexes of each band were digested with trypsin as described before. The samples were dried in a vacuum concentrator and reconstituted in 100 μl of 0.1% formic acid. 4.5 μl of the resulting peptide mixture was analyzed in LTQ Velos Orbitrap. The MS analysis were performed as described before, except the instrument methods in LTQ Velos Orbitrap were set up in the data-dependent acquisition mode of higher-energy collisional dissociation fragmentation. The resolution in the Orbitrap system was set to r = 60,000, and the five most intense peptide ions with charge states of ≥2 were sequentially isolated to a target value of 50,000 and fragmented in higher-energy collisional dissociation with normalized collision energy of 40% with the resolution in the Orbitrap system was set to r = 7,500 for MS/MS. The signal threshold for triggering an MS/MS event was set to 80,000 counts, and an activation time of 0.1 ms was used. Dynamic exclusion was enabled with an exclusion size list of 400, an exclusion duration of 60 s, and a repeat count of 2. For protein identification, peak lists (msf) were generated from the raw data files using Proteome Discoverer version 1.3 (Thermo Fisher Scientific) with Sequest search engine and searched against Human International Protein Database, version 3.86 (91,522 sequences; 36,630,302 residues, release July 2011) with carbamidomethylation (+57.021 Da) as fixed modification, oxidation of methionine (+15.995 Da), and chemical cross-linked with DSS (mass of dead-end cross-linking, 156.07864 Da) as variable modifications, one trypsin missed cleavage, and a tolerance of 10 ppm for precursor and 0.02 Da for fragment ions.

For cross-linked analysis, the raw data files generated by Xcalibur version 2.1 (Thermo Fisher Scientific) were converted to a peak list format (mgf) using Proteome Discoverer version 1.3 (Thermo Fisher Scientific). The mgf files were analyzed in MassMatrix software (34) to automatically search chemical cross-linkage against databases containing the ADAM17cyto and Trx-1 amino acid sequences, according to the software instructions. The parameters for cross-linking analysis used in
MassMatrix software were carbamidomethylation (+57.021 Da) as fixed modification, oxidation of methionine (+15.995 Da) as variable modifications, chemical cross-linked with DSS (138.06808 Da) noncleavable by enzymes, four trypsin missed cleavages, and a tolerance of 10 ppm for precursor and 0.02 Da for fragment ions. Search results with high confidence (MassMatrix pp score) and potential cross-linked peptides were manually validated for b and y ion series containing and chains (35, 36). This experiment was performed three times.

In Silico Analysis—A high quality homology model of the human Trx-1 was built using multiple templates found on the Protein Data Bank with high identity and 100% coverage using Yasara (37). To elucidate the interacting region, an ab initio model of the ADAM17 cytoplasmic domain was obtained from I-Tasser (38) and evaluated by C-Score and TM-Score (39) approaches. A customized docking algorithm was developed through Rosetta (40) using cross-link information as constraints of the search space. The resulting decoys were assayed on energy values and lysine pair distance. Low energy models were validated through an automated DSS bonding protocol generating a final theoretical model of the interaction interface. Binding energy contributions of each interface residue and protein stability were calculated using an alanine scanning to evaluate only the interface residues of ADAM17 for a 10-ns molecular dynamics simulation with explicit solvent using Yasara. Each 25 ps, a snapshot was extracted, and each interface residue was mutated to alanine. Then the whole complex had its side chains optimized using both a standard rotamer library and the force field parameters. For each mutation the binding energy of Trx-1 to wild-type ADAM17 is compared with mutated ADAM17. The difference corresponded to the level of importance of the mutated residue for the interaction interface. The binding energy function used in the calculations considered, besides the interaction potential, the solvation effects given by

FIGURE 1. Thioredoxin-1 is revealed as novel ligand of ADAM17. HEK293 cells were transfected with FLAG-ADAM17 cyto; after 48 h the intracellular proteins were collected and submitted to IP followed by MS and immunoblotting (IB). A, the first of the three panels shows the IB of the FLAG-tagged ADAM17 cytoplasmic domain and the negative control after IP confirming the protein expressions. Then it shows the input of protein lysates and the detection of endogenous Trx-1 in both conditions. Finally, it confirms the Trx-1 interaction with FLAG-ADAM17cyto by IB. Arrows indicate the identification of Trx-1 bands, and asterisks indicate unspecific bands. The lower panel shows the expression of full-length ADAM17-HA and its negative control by IB. It also demonstrates that the endogenous Trx-1 interacts with full-length ADAM17-HA by IP followed by IB. B, manual validation of Trx-1 peptide, spectra containing b and y ion series. C, confocal immunofluorescence confirmed the co-localization between endogenous Trx-1 and full-length ADAM17-HA (upper panels) and likely endogenous Trx-1 and FLAG-ADAM17cyto (lower panels) in HeLa cells. Prot G, protein G.
**TABLE 2**

| Proteins (residues) | Peptide sequence | m/z range | XIC of peptide from Me2SO normalized | XIC of peptide from PMA treatment | PMA/Me2SO normalized |
|---------------------|------------------|-----------|--------------------------------------|-----------------------------------|----------------------|
| ADAM17 (739–753)    | LQPAPIVPSAAPK    | 728.9224–728.9448 | 1.42 × 10^4                          | 1.11 × 10^4                        | 0.78                 |
| ADAM17-Ser791       | SFEDLTDHPVTR     | 708.8350–708.8500 | 6.54 × 10^4                          | 8.00 × 10^4                        | 1.22                 |
| ADAM17-Ser979       | pSFEDLTDHPVTR    | 748.8170–748.8320 | 1.99 × 10^4                          | 3.72 × 10^4                        | 0.18                 |
| ADAM17-Ser115       | VDSKETEC         | 484.1860–484.1942 | 7.65 × 10^4                          | 1.76 × 10^4                        | 2.3                  |
| ADAM17-Ser119       | VDPKETEC         | 524.1830–524.1950 | 1.51 × 10^4                          | 2.39 × 10^4                        | 15.8                 |
| Trx-1 (9–21)        | TAFQALDDADGDK    | 668.8130–668.8300 | 1.78 × 10^4                          | 1.68 × 10^4                        | 0.94                 |

# Analysis of ADAM17 Activity on AP Reporter Assay—
HEK293 cells stably transfected with HB-EGF-AP were seeded into 100-mm dishes and co-transfected with transient Trx-1-HA and empty vector (negative control). After 48 h, the cells were trypsinized, counted to 3 × 10^5 cells/well, and seeded in 24-well plates (Corning). The following day, the cells were starved for 4 h and activated with PMA (50 ng/ml) or EGF (100 ng/ml) (3) for 1 h in a phenol-free medium. The cleavage of HB-EGF-AP was measured after overnight incubation. Briefly, 100 μl of conditioned media were collected of each well and added to individual wells of a 96-well plate containing 100 μl of AP buffer. The AP ratio was calculated as previously described (3). Three independent experiments were performed in duplicate for PMA activation and in triplicate for EGFP activation.

To confirm that ADAM17 is the sheddase responsible for PMA-induced HB-EGF cleavage, WT and Adam17^-/-^ mEF cells expressing HB-EGF-AP were activated by PMA as described above. The conditioned medium was incubated with AP buffer. To assess the amount of total HB-EGF-AP expressed, the cells were collected, and the lysates were also incubated with AP buffer. The AP ratio was calculated as previously described (3). Three independent experiments were performed in triplicate.

To indirectly evaluate the effect of Trx-1 overexpression on HB-EGF-AP levels, the membrane proteins of HEK293 cells co-transfected with Trx-1-HA were collected by the enrichment of membrane protein protocol (27). The proteins were separated by a 12% SDS-PAGE under nondenaturing conditions, and the AP activity was assessed onto nitrocellulose membrane after incubation with BCIP/NBT (Sigma), according to the manufacturer’s protocol.

**Analysis of mRNA Expression Levels**—To analyze the mRNA expression of Trx-1, ADAM17, and HB-EGF, HEK293 cells stably expressing HB-EGF-AP were co-transfected with Trx-1-HA, as described above. Total RNA was obtained using the TRIzol reagent (Invitrogen), and 3 μg of total RNA were used for retro-transcription using a first strand cDNA synthesis kit (GE Healthcare). Real time quantitative PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems), and the dissociation curves were performed to confirm the specificity of products. The primers sequences were: Trx-1, 5’-GGACGCCTGCAAGGTGATAAACTTGTGTTGCATTCACACTC-3’ and 5’-TTGCG-TGCATTTGACCTTTGCAATGACC-3’; ADAM17, 5’-GGACCCTTTCCCCAAATAAGCAGC-TCAAA-3’ and 5’-ATGTTCCCTGAGATCCTTGAGT-TCTCT-3’ and 5’-ACTGTATCCACGGACAGCTGCTATA-3’. The threshold cycles (Ct) values of target genes were normalized relative to the GAPDH gene, and relative expression ratios were calculated by the 2–ΔΔCt method. Three independent experiments were performed in triplicate.

**Statistical Analysis**—For the statistical analysis of HEK293 AP assays, we performed one-way ANOVA followed by Tukey test. For mEF cells AP assays, we used one-way ANOVA followed by a Bonferroni test to compare within each WT and Adam17^-/-^- mEF cells (control versus PMA). The significance level was stated at 0.05 (GraphPad Prism version 5 for Windows).

**RESULTS**

Identification of Thioredoxin-1 as ADAM17 Cytoplasmic Domain Interaction Partner by LC-MS/MS—To identify the interaction partners of the intracellular ADAM17 domain, the recombinant protein was expressed in HEK293 cells (Fig. 1A, left panel). After IP, the complexes were eluted with FLAG peptide, digested with trypsin, and analyzed by LC-MS/MS. Three experiments were performed with a negative control (FLAG-GFP recombinant protein). Among the proteins found, we identified Trx-1 only in the IP with FLAG-ADAM17cyto compared with the negative control. One peptide of Trx-1 was manually validated (Fig. 1B).

Validation of Thioredoxin-1 as a Cytoplasmic Domain Partner by Immunoblotting—To validate the interaction between FLAG-ADAM17cyto and Trx-1, an aliquot of immunoprecipitation complexes with FLAG-ADAM17cyto and negative control (FLAG-GFP recombinant protein) were separated by SDS-PAGE and analyzed by immunoblotting. This result showed the presence of endogenous Trx-1 only in the IP with FLAG-ADAM17cyto (Fig. 1A, right panel). Furthermore, in the IP using full-length ADAM17-HA, Trx-1 was also identified by immunoblotting (Fig. 1A, lower panel).

Thioredoxin-1 Co-localizes with Cytoplasmic Domain and Full-length ADAM17 Recombinant Proteins in HeLa Cells—After the validation of the interaction between Trx-1 and FLAG-ADAM17cyto, we evaluated whether the proteins co-localize. The recombinant proteins, FLAG-ADAM17cyto and full-length ADAM17-HA, were transiently expressed in HeLa cells, and confocal immunofluorescence analysis showed that both proteins were co-localized with endogenous Trx-1 (Fig. 1C).
Thioredoxin-1 Interaction Is Independent of the Phosphorylation of ADAM17 Residues: Ser791 or Ser819—To evaluate whether the endogenous Trx-1 interaction is dependent or independent of phosphorylation, we overexpressed FLAG-ADAM17cyto in HEK293 cells, and the cells were treated with PMA. After IP, eluted complexes were digested with trypsin and analyzed by LC-MS/MS. By XIC analysis, we observed that the PMA treatment resulted in a lower phosphorylation of Ser(P)791 and a higher phosphorylation of Ser(P)819 than the control cells, considering that the ratio between XIC of phosphorylated peptides in the presence of PMA and Me2SO was higher than 1.5-fold change (Table 2). However, Trx-1 was found in both preparations with the same abundance (Table 2). The spectrum of phosphorylated peptide was manually validated, confirming the phosphorylation sites at Ser(P)791 and Ser(P)819 (Fig. 2).

ADAM17 Cytoplasmic Domain Interacts with Trx-1 by Solid Phase Binding Assays—We demonstrated that the interaction between His-FLAG-ADAM17cyto and His-Trx-1-HA recombinant proteins occurs in a concentration-dependent manner by immobilizing either Trx-1 or ADAM17cyto (Fig. 3, A and B, respectively).

ADAM17 Cytoplasmic Domain Interacts with Trx-1 by NMR—To evaluate the interaction between Trx-1 and ADAM17cyto, the proteins were expressed in bacteria, purified by affinity chromatography and analyzed by NMR. The NH signals of the main chain protein (His-Trx-1-HA) that showed marked changes in their chemical shifts frequencies during the titration with the...
binding protein (His-FLAG-ADAM17cyto) were used to confirm the interaction between the proteins. These signals correspond to the labeled protein amino acids that interacted with the unlabeled protein (Fig. 4).

**Chemical Cross-linking Coupled with Mass Spectrometry**—To evaluate the interaction between Trx-1 and FLAG-ADAM17cyto, we chemically cross-linked the recombinant purified proteins. Initially, the cross-linked proteins (His-FLAG-ADAM17cyto and His-Trx-1-HA) were separated by SDS-PAGE, and the three main bands of 170, 55, and 34 kDa were digested with trypsin. After manually verifying possible hits from MassMatrix search results, we identified one cross-link with high confidence (MassMatrix pp score $\geq 30$). The 170-kDa band showed the identification of cytoplasmic domain and Trx-1 complexes by tryptic peptides $^{726}$IKPFPAPQTPGR$^{738}$ and $^{82}$KGQ$^{85}$, respectively, cross-linked by the side chains of the underlined lysine residues. This experiment was repeated three times, and the same cross-linked peptides were confirmed. The validation of cross-linked peptides is shown in the Fig. 5A.

**In Silico Analysis Proposes a Model for the Interaction**—Our homology model of the human Trx-1 (Fig. 5C) presented a RMSD of 1.089 Å compared with the chimeric human and *Escherichia coli* protein (Protein Data Bank code 1M7T) and an overall Z-Score of 1.008, which indicated a good quality for the model. The *ab initio* model showed a C-Score of 2.59 and a TM-Score of 0.41 ± 0.14. Our studies suggest that the ADAM17 cytoplasmic domain is very flexible and is presented here in an acceptable topology. The custom docking algorithm resulted 5,000 decoys that were filtered by binding energy and compatible cross-link lysine pair distance (Fig. 5, B and C). Best fitted model after the molecular dynamics simulation exhibited an overall mean Ca-RMSD of 3 Å, and further analysis demonstrated the high flexibility of the ADAM17 cytoplasmic domain with a relative mean RMSD of 5.722 Å when compared with Trx-1 (Fig. 5D). Distribution of differences of binding energy suggests the contribution of each interface residue of the cytoplasmic domain of ADAM17 (Fig. 5E). Residues with averages above zero showed an indication that mutation disturbed the interaction surface and thus must be important to keep the complex in the bound configuration.

**The Increase of Trx-1 Expression Modulates HB-EGF Shedding upon Activation of ADAM17 with PMA**—After the characterization of the interaction between Trx-1 and FLAG-ADAM17cyto, we evaluated the effect of overexpression of Trx-1 on ADAM17 activity. For that, HEK293 cells stably expressing HB-EGF-AP were transiently transfected with pcDNA-Trx-1-HA and treated with PMA. By analysis of AP activity in the conditioned media of the cells, HEK293 overexpressing Trx-1 showed lower HB-EGF shedding compared with control cells (Fig. 6A, $p < 0.05$, ANOVA followed by Tukey test). In addition, using WT and *Adam17*−/− mEF cells, we confirmed ADAM17 is the sheddase responsible for PMA induced HB-EGF cleavage (Fig. 6G, $p < 0.05$, ANOVA followed by Bonferroni test). The expression of Trx-1 in the same experimental conditions was confirmed by real time quantitative PCR (Fig. 6B). Furthermore, we demonstrated that Trx-1-HA overexpression did not affect the HB-EGF-AP expression on cell surface or the mRNA levels (Fig. 6, C and D, respectively);
Thioredoxin-1 Is a Novel Interaction Partner of ADAM17

ADAMs are one of the main proteases responsible for the ectodomain shedding of surface membrane proteins; however, how membrane-anchored proteinases can be activated by inside-out regulation remains unclear. To identify the interaction partners of the cytoplasmic domain and to correlate them with a functional role in ADAM17 activation, we induced the expression of the ADAM17 cytoplasmic domain in HEK293 cells and identified the binding partners using mass spectrometry.

We found thioredoxin-1 as a ligand of the ADAM17 cytoplasmic domain by MS and immunoblotting (Fig. 1, A and B). The immunoprecipitation data confirmed that full-length ADAM17 also interacts with Trx-1, which suggests that the interaction is not restricted to the soluble cytoplasmic recombinant protein (Fig. 1A, lower panel). Co-localization assays further validated the interaction, showing that endogenous Trx-1 co-localizes with the ADAM17 cytoplasmic domain and full-length ADAM17 recombinant proteins (Fig. 1C, upper and lower panels, respectively).

ADAM cytoplasmic domains are kinase targets (2, 6, 42), so we evaluated whether the phosphorylation state could interfere in Trx-1 binding. We found that the Trx–1 interaction with the cytoplasmic domain is not dependent on Ser(P)791 and Ser(P)819 because changes in the phosphorylation pattern induced by PMA treatment (42), such as the decrease in Ser(P)791 and the increase in Ser(P)819, did not change the abundance of Trx-1 binding (Table 2).

To evaluate the interaction of Trx-1 and ADAM17 cytoplasmic domain, we used three different approaches: solid phase binding assay, NMR, and chemical cross-linking coupled with MS. The solid phase binding assays demonstrated that the interaction between the proteins occurs in a concentration-dependent manner (Fig. 3). From NMR studies, we also determined that the presence of the ADAM17 cytoplasmic domain promotes perturbations in the conformational dynamics of the Trx-1 (Fig. 4). The MS analysis of the complexes showed the interaction between the two proteins to be cross-linked by the side chains of the lysine residues (Fig. 5A) in the tryptic peptides of ADAM17 cytoplasmic domain and Trx-1, \(72^6\text{IKPFPAPQPPTGK}^{738}\) and \(82^\text{KGQK}\), respectively. As previously reported by Willems et al. (7), the interaction of thiol isomerases is rapid and not very stable in the reduced form; therefore, this approach that stabilized the complex by chemical cross-linking in vitro made it possible to confirm this interaction through the side chain of lysine residues that showed at least a 11.4 Å of distance (based on the DSS spacer arm). In addition, based on the in silico analysis of the protein–protein interaction between ADAM17 cytoplasmic domain and Trx-1 (Fig. 5B), it can be observed that the active site residues of Trx-1 are not in contact with the interface region of ADAM17 cytoplasmic domain and are free for binding to different substrates and to thereby perform different activities. Despite the high flexibility of ADAM17 cytoplasmic domain of ADAM17, the interface region is kept along molecular dynamic simulation. Residues neither alters the mRNA levels of ADAM17 (Fig. 6E) nor the pro and mature forms of ADAM17 (Fig. 6F).

The Increase of Trx-1 Expression Modulates HB-EGF Shedding upon Activation of ADAM17 with EGF—We demonstrated that the activation of ADAM17 with EGF is decreased in the presence of Trx-1 (Fig. 7, \(p < 0.05\), ANOVA followed by Tukey test).

DISCUSSION

The Increase of Trx-1 Expression Modulates HB-EGF Shedding upon Activation of ADAM17 with EGF—We demonstrated that the activation of ADAM17 with EGF is decreased in the presence of Trx-1 (Fig. 7, \(p < 0.05\), ANOVA followed by Tukey test).
that contribute considerably to the binding in ADAM17 cytoplasmic domain are observed to be above zero (Fig. 5E).

Trx-1 is known to act as a reductase via a dithiol/disulfide exchange reaction between two cysteine residues in the active site, Cys\textsuperscript{32}-Gly-Pro-Cys\textsuperscript{35}, on oxidized protein substrates that typically contain disulfide bonds. Trx-1 can also be found to be more oxidized when additional disulfide bonds are formed (43). ADAM17 has two CXXC motifs, but only in the disintegrin and cysteine-rich domains (44, 45), and these motifs could be reduced by extracellular Trx-1.

Previous studies have shown that intracellular Trx-1 plays a crucial role in the ROS scavenging (43, 46) and that ROS and

**FIGURE 6. Thioredoxin-1 modulates HB-EGF shedding in HEK293 cells under PMA stimulation.** HEK293 cells stably expressing HB-EGF-AP were transiently transfected with Trx-1 recombinant protein. After 48 h, the cells were submitted to a starvation period of 4 h followed by PMA activation for 1 h. Supernatant was collected and evaluated by AP assay. A, AP reporter assay indicates that Trx-1 decreases the HB-EGF shedding in HEK293 cells after PMA treatment. Three independent experiments were performed in duplicate (one-way ANOVA followed by Tukey test. Different letters indicate statistically difference at p < 0.05). B, real time quantitative PCR confirms the expression of Trx-1-HA after transient transfection in the same experimental conditions used before. C, alkaline phosphatase activity indirectly shows HB-EGF expression after the enrichment of membrane proteins from HEK293 cells stably expressing HB-EGF-AP and transiently expressing Trx-1-HA. Densitometry of the alkaline phosphatase substrate bands are shown in the right panel. D, the expressions of HB-EGF and Trx-1 in HEK293 cells are also confirmed by real time quantitative PCR. E and F, the increase in the expression of Trx-1 does not change the ADAM17 mRNA levels (E) and likely the proADAM17 and its mature form after membrane protein enrichment in HEK293 cells (F). G, to confirm ADAM17 is the sheddase responsible for PMA induced HB-EGF cleavage, the WT and Adam17/−/− mEF cells were transiently transfected with the vector encoding HB-EGF-AP and activated by PMA. AP ratio was calculated between the supernatant AP activity and the total AP activity in the cell lysate plus supernatant from three identically prepared wells and averaged (3). Three independent experiments were performed in triplicate (one-way ANOVA followed by Bonferroni test for selected pairs of columns). * statistical difference at p < 0.05.
thiol isomerases are involved in the regulation of ADAM proteolytic activity (7–10, 47–50). Therefore, we investigated whether ADAM17 activity could be modulated by Trx-1 using a standard approach in a cell model of HB-EGF shedding coupled with an AP reporter assay (7, 25). We found that the transient overexpression of Trx-1-HA in HEK293 cells treated with PMA or EGF negatively modulated the activity of ADAM17 (Figs. 6 and 7, respectively). The presence of ROS scavengers or the inhibition of cell surface oxidoreductases has previously been shown to prevent HB-EGF cleavage and LPS-induced ADAM17 activity (7, 10). Furthermore, we may also consider that ADAM17 cytoplasmic domain can solely function as an anchor domain to recruit Trx-1, which can regulate the redox state of cysteine residues in target proteins near or in the membrane, for instance in lipid rafts (41, 50). In summary, the present study has demonstrated that Trx-1 is a novel interaction partner of the ADAM17 cytoplasmic domain and suggests that Trx-1 is a candidate that could be involved in the regulation of ADAM17 activity.

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