The TspanC8 subgroup of tetraspanins interacts with a disintegrin and metalloprotease 10 (ADAM10) and regulates its maturation and cell surface expression

Haining, Elizabeth; Yang, Jing; Gavin, Rebecca; Khan, Kabir; Collier, Richard; Tsai, Schickwann; Watson, Steve; Frampton, Jonathan; Garcia, Paloma; Tomlinson, Michael

DOI: 10.1074/jbc.M112.416503

License: None: All rights reserved

Citation for published version (Harvard):
Haining, EJ, Yang, J, Bailey, RL, Khan, K, Collier, R, Tsai, S, Watson, SP, Frampton, J, Garcia, P & Tomlinson, MG 2012, 'The TspanC8 subgroup of tetraspanins interacts with a disintegrin and metalloprotease 10 (ADAM10) and regulates its maturation and cell surface expression' Journal of Biological Chemistry, vol. 287, no. 47, pp. 39753-39765. DOI: 10.1074/jbc.M112.416503
Cell Biology:
The TSPAN C8 Subgroup of Tetraspanins Interacts with A Disintegrin and Metalloprotease 10 (ADAM10) and Regulates Its Maturation and Cell Surface Expression

Elizabeth J. Haining, Jing Yang, Rebecca L. Bailey, Kabir Khan, Richard Collier, Schickwann Tsai, Steve P. Watson, Jon Frampton, Paloma Garcia and Michael G. Tomlinson

Access the most updated version of this article at doi: 10.1074/jbc.M112.416503

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2012/10/03/M112.416503.DC1.html

This article cites 45 references, 22 of which can be accessed free at http://www.jbc.org/content/287/47/39753.full.html#ref-list-1
The TspanC8 Subgroup of Tetraspanins Interacts with a Disintegrin and Metalloprotease 10 (ADAM10) and Regulates Its Maturation and Cell Surface Expression*†‡¶

Received for publication, February 24, 2012, and in revised form, September 30, 2012 Published, JBC Papers in Press, October 3, 2012, DOI 10.1074/jbc.M112.416503

Elizabeth J. Haining†, Jing Yang†, Rebecca L. Bailey†, Kabir Khan†, Richard Collier†, Schickwann Tsai‡, Steve P. Watson†, Jon Frampton‡, Paloma Garcia‡, and Michael G. Tomlinson‡

From the †School of Biosciences, College of Life and Environmental Sciences and Schools of ¶Clinical and Experimental Medicine and §Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, United Kingdom and ‡Division of Hematology, Department of Medicine, University of Utah, Salt Lake City, Utah 84132

A disintegrin and metalloprotease 10 (ADAM10) is a ubiquitous transmembrane metalloprotease that cleaves the extracellular regions from over 40 different transmembrane target proteins, including Notch and amyloid precursor protein. ADAM10 is essential for embryonic development and is also important in inflammation, cancer, and Alzheimer disease. However, ADAM10 regulation remains poorly understood. ADAM10 is compartmentalized into membrane microdomains formed by tetraspanins, which are a superfamilly of 33 transmembrane proteins in humans that regulate clustering and trafficking of certain other transmembrane “partner” proteins. This is achieved by specific tetraspanin-partner interactions, but it is not clear which tetraspanins specifically interact with ADAM10. The aims of this study were to identify which tetraspanins interact with ADAM10 and how they regulate this metalloprotease. Co-immunoprecipitation identified specific ADAM10 interactions with Tspan5, Tspan10, Tspan14, Tspan15, Tspan17, and Tspan33/Penumbra. These are members of the largely unstudied TspanC8 subgroup of tetraspanins, all six of which promoted ADAM10 maturation. Different cell types express distinct repertoires of TspanC8 tetraspanins. Human umbilical vein endothelial cells express relatively high levels of Tspan14, the knockdown of which reduced ADAM10 surface expression and activity. Mouse erythrocytes express predominantly Tspan33, and ADAM10 expression was substantially reduced in the absence of this tetraspanin. In contrast, ADAM10 expression was normal on Tspan33-deficient mouse platelets in which Tspan14 is the major TspanC8 tetraspanin. These results define TspanC8 tetraspanins as essential regulators of ADAM10 maturation and trafficking to the cell surface. This finding has therapeutic implications because focusing on specific TspanC8-ADAM10 complexes may allow ADAM10 therapeutic targeting in a cell type- and/or substrate-specific manner.

The proteolytic cleavage, or “shedding,” of the extracellular regions (ectodomains) of transmembrane proteins is emerging as an important mechanism for the regulation of cell development and function (1–3). A disintegrin and metalloprotease 10 (ADAM10)† is a type 1 transmembrane protein that is ubiquitously expressed and functions as an ectodomain sheddase for over 40 transmembrane target proteins (4). ADAM10-deficient mice die at embryonic day 9.5, consistent with the essential role of ADAM10 in the cleavage and activation of Notch, which is central to most cell fate decision-making processes during metazoan development (5). Amyloid precursor protein is an ADAM10 target that is central to Alzheimer disease (6). Cleavage of amyloid precursor protein by β- and γ-secretases gives rise to the pathogenic amyloid β-peptide that forms the amyloid plaques in the brains of Alzheimer disease patients. Counteracting this is the α-secretase activity of ADAM10 that cleaves amyloid precursor protein at a third site, thus preventing release of the pathogenic peptide. Indeed, transgenic overexpression of ADAM10 prevents amyloid plaque deposition in a mouse model of Alzheimer disease, suggesting that ADAM10 is a promising target for disease treatment in humans (6). ADAM10 targets in the vasculature include the platelet-activating collagen receptor glycoprotein VI (7, 8) and endothelial proteins with roles in angiogenesis and inflammation, such as vascular endothelial growth factor receptor 2 (9), the junctional

* This work was supported by two Biotechnology and Biological Sciences Research Council studentships (to E. J. H. and R. L. B.), a British Heart Foundation (BHF) chair (to S. P. W.), a Science City Research Alliance fellowship (to P. G.), and BHF Senior Research Fellowship FS/08/062/25797 (to M. G. T.).
† This article contains supplemental Figs. I–V.
‡ To whom correspondence should be addressed. Tel: 44-121-414-2507; Fax: 44-121-414-5925; E-mail: m.g.tomlinson@bham.ac.uk.
adhesion molecule VE-cadherin (10), and transmembrane chemokines CX3CL1 and CXCL16 (11). Despite the importance of ADAM10, the regulation of its trafficking, activation, and localization to targets is poorly understood. Mechanistic characterization of these events is essential if we are to harness the potential of ADAM10 as a therapeutic target.

The regulation of transmembrane proteins by their compartmentalization into membrane microdomains is a concept that has developed over recent years through the study of neuronal and immunological synapses, lipid rafts, caveolae, and tetraspanins. The latter are a superfamily of four-transmembrane proteins that number 33 in humans (12, 13). Tetraspanins possess two extracellular regions, the larger of which has four to eight conserved cysteine residues that form structurally important disulfide bonds. They appear to fold into compact rod-shaped structures that only project about 5 nm from the plasma membrane and play a fundamental organizing role by self-associating to form microdomains. Specific tetraspanins recruit so-called “partner” proteins into the microdomains. These partners include certain integrins, immunoglobulin superfamily proteins, and proteases, and in some cases, their interaction with tetraspanins is essential for normal biosynthesis and trafficking to the plasma membrane (12, 13). Tetraspanin microdomains can also facilitate cell adhesion and signaling by providing platforms in which associated proteins can be clustered. For example, the endothelial cell adhesion molecules ICAM-1, VCAM-1, and P-selectin require clustering by tetraspanins CD9, CD151, and CD63, respectively, for efficient capture of leukocytes from the circulation (14, 15). In addition, Frizzled-4 appears to require clustering by tetraspanin Tspan12 to allow appropriate β-catenin signaling for development of the retinal vasculature in mice and humans (16–18). In contrast, vascular development in mice is normal in the absence of CD151, but pathological angiogenesis is defective, possibly due to the well-characterized regulation of laminin-binding integrins α3β1 and α6β1 by this tetraspanin (19). On platelets, CD151 and Tspan32 are essential for thrombus formation in vivo (20, 21), and Tspan33 (previously named Penumbra) is required for normal erythropoiesis (22), although the mechanisms responsible for these phenotypes remain unclear.

ADAM10 was recently shown to predominantly localize to tetraspanin microdomains in human leukocyte cell lines (23). In addition, tetraspanin antibodies could promote ADAM10 activity as measured by shedding of two ADAM10 targets, TNF-α and EGF (23). A second study showed that ADAM10 co-immunoprecipitated with Tspan12 in human breast cancer and neuroblastoma cell lines (24). Tspan12 also promoted ADAM10 maturation, which involves the cleavage of its prodomain by proprotein convertases during trafficking to the plasma membrane. Moreover, ADAM10 activity was enhanced by Tspan12 as detected by shedding of the ADAM10 target amyloid precursor protein (24). However, Tspan12 is unlikely to be the only tetraspanin that regulates ADAM10 because it does not share the ubiquitous expression profile of the metalloprotease, instead appearing relatively restricted to endothelial cells of the retina, meninges of the brain, and smooth muscle cells of the colon (16). Indeed, Tspan12 does not appear to be expressed by the platelet (25–29), a cell type on which we chose to initially focus because it expresses many other tetraspanins as well as ADAM10. Therefore, in the present study, we tested the hypothesis that one or more platelet tetraspanins could interact with and regulate ADAM10. This led to the identification of a subgroup of six related, but largely unstudied, tetraspanins that fulfilled these criteria that we termed the TspanC8 tetraspanins due to the eight cysteine residues within their main extracellular region. These tetraspanins appear to play a common role in promoting ADAM10 maturation and trafficking to the plasma membrane.

**Experimental Procedures**

**Antibodies**—Mouse anti-human mAbs were 11G2 (23) and 163003 (R&D Systems) to ADAM10, 11B1 to CD151 (30), IV.3 to FcγRIIa (Medarex), and 1.3 to PECAM-1 (a gift from Peter Newman, Wisconsin, MI). The rat anti-mouse ADAM10 mAb was 139712 (R&D Systems), control mouse IgG1 was MOPC-21 (Sigma), and control rat IgG2A was 54447 (R&D Systems). Polyclonal antibodies were the AB936 goat anti-human ADAM10 (R&D Systems) and sc-6458 goat anti-VE-cadherin C terminus (Santa Cruz Biotechnology). Anti-epitope tag antibodies were mouse anti-FLAG mAb M2 and rabbit anti-FLAG (Sigma), rabbit anti-HA mAb C29F4 (Cell Signaling Technology), and mouse anti-GFP mAb 3E1 (Cancer Research UK).

**Expression Constructs**—Tetraspanin expression constructs with N-terminal FLAG tags were generated by cloning tetraspanin cDNAs into the pEF6-FLAG vector (27), which is a modified version of pEF6/Myc–His A (Invitrogen). Tetraspanin constructs with N-terminal GFP tags were similarly made using the pGFP-C vector (BioSignal Packard). Tetraspanin cDNAs were generated by PCR from cDNA derived from the HEK293T cell line, primary mouse megakaryocytes, or megakaryocyte-like cell line L8057. Palmitoylation mutants of human tetraspanins were generated by a two-PCR strategy (31) that mutated all cysteine residues at the membrane-cyttoplasm interface to serines. These were amino acids 13, 80, 89, and 95 for Tspan14; 12, 101, 287, 288, and 290 for Tspan15; and 81, 83, 92, and 100 for Tspan33. The HA-tagged bovine ADAM10 in the pcDNA3 vector has been described previously (32).

**Mice**—Tspan33/Penumbra knock-out mice have been described previously (22). All animal maintenance and experiments had appropriate Home Office approval and licensing according to Animals (Scientific Procedures) Act 1986. Knock-out mice and littermate controls were generated by breeding heterozygote animals. All animals used for experiments were aged 8 weeks or older.

**Cell Culture and Transfection**—All cell culture reagents were from PAA unless stated. The human embryonic kidney (HEK)-293T (HEK-293 cells expressing the large T-antigen of simian virus 40) cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfection of HEK-293T cells was carried out using polyethylenimine (Sigma) as described (33). The A549 human lung epithelial cell line was cultured in the same medium as HEK-293T cells. Primary human umbilical vein endothelial cells (HUVECs) were provided by Phil Stone and Gerard Nash (Uni-
cell surface biotinylation experiments were conducted as described (27). For mouse erythrocyte surface biotinylation experiments, whole blood obtained by exsanguinations (35) was centrifuged at 200 × g for 5 min, and the erythrocytes were separated from the platelet-rich plasma layer. Erythrocytes were washed with modified Tyrode’s buffer containing 5% acid citrate dextrose (35). The cells were biotinylated with 2 mM EZ-Link sulfo-N-hydroxysuccinimide long chain biotin (Thermo Scientific) for 30 min and quenched with 0.1 M glycine. Subsequent cell lysis, immunoprecipitations, and Western blotting were as described (27). For ADAM10 stability studies, A549 cells were transfected with siRNAs and 3 days later were biotinylated with 1 mM EZ-Link sulfo-NHS-LC-biotin (Thermo Scientific) for 30 min and quenched with 0.1 M glycine. Some cells were harvested and frozen immediately, whereas others were returned to culture and harvested and frozen at 24, 36, or 48 h postbiotinylation. Cell pellets were lysed in 1% Triton X-100 lysis buffer (27) containing a protease inhibitor mixture (Sigma). Following centrifugation to remove nuclei and insoluble debris, lysates were subjected to mouse anti-ADAM10 or control MOPC-21 immunoprecipitation for 90 min. The immunoprecipitates were washed with 1% Triton X-100 lysis buffer, separated by SDS-PAGE on non-reducing gels, and Western blotted with IRDye fluorescent neutraavidin (LI-COR Biosciences). The results were imaged and quantified on the Odyssey Infrared Imaging System (LI-COR Biosciences).

Cell Surface Chemical Cross-linking Experiments—Two days following transfection with HA-ADAM10 and FLAG-tetraspanins, HEK-293T cells were washed with PBS and treated with a 2 mM concentration of the cell surface cross-linker 3,3′-dithiodi-sulfo succinimidylpropionate (DTSSP) from Thermo Fisher Scientific for 30 min on ice. The cross-linking was quenched with 0.1 M glycine, and the cells were scraped off and lysed in 1% Triton X-100 lysis buffer (27) containing a protease inhibitor mixture (Sigma). Following centrifugation to remove nuclei and insoluble debris, lysates were supplemented with 0.1% SDS to increase lysis buffer stringency, and mouse anti-FLAG immunoprecipitations were performed overnight using protein G-Sepharose beads (Invitrogen), or anti-GFP immunoprecipitations were performed overnight using GFP-Trat® beads (ChromoTek). The immunoprecipitates were washed with 1% Triton X-100, 0.1% SDS lysis buffer and separated by SDS-PAGE on reducing gels to break the disulfide bond within DTSSP, thus allowing co-immunoprecipitated proteins to run at their predicted molecular weights. Western blotting was carried out with anti-HA and anti-FLAG or anti-GFP primary antibodies and specific IRDye fluorescent secondary antibodies (LI-COR Biosciences). The results were imaged and quantified on the Odyssey Infrared Imaging System (LI-COR Biosciences).

Digitonin Lysis Experiments—These were done in a manner similar to the experiments in the previous section except that cells were not subjected to DTSSP cross-linking but were instead directly lysed in 1% digitonin (Merck Millipore) lysis buffer (38) containing a protease inhibitor mixture (Sigma). Immunoprecipitations were done for 90 min rather than over-night and were washed in 1% digitonin lysis buffer.
VE-cadherin Cleavage Assay—This assay was performed as described (10) using HUVECs 3 days post-siRNA transfection. The γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (Sigma) at 10 μM was added 18 h before harvesting the cells to prevent further proteolysis of VE-cadherin after ADAM10 cleavage. The results were imaged and quantified on the Odyssey Infrared Imaging System (LI-COR Biosciences).

Flow Cytometry—For ADAM10 flow cytometry analyses of mouse platelets and erythrocytes, whole blood was obtained by exsanguinations (35), and 5-μl volumes were stained in 50 μl of PBS, 1 mM EGTA with 10 μg/ml FITC-conjugated rat anti-mouse ADAM10 mAb or negative control FITC-conjugated rat IgG2A. Platelets and erythrocytes were analyzed using a FACSCalibur (BD Biosciences) by first gating the cells according to size. The level of ADAM10 expression on each cell type was then calculated by subtracting the negative control geometric mean fluorescence intensity from the specific ADAM10 value. ADAM10 flow cytometry analyses of HUVECs were performed by first scraping off the cells and then staining 1 × 10^5 cells with 10 μg/ml FITC-conjugated mouse anti-human ADAM10 mAb or negative control FITC-conjugated mouse IgG1. Following FACSCalibur analysis, the level of ADAM10 expression was calculated as described above.

Quantitative PCR—RNA was isolated using the Norgen Biotek Total RNA Purification kit and used to make cDNA using the Invitrogen High Capacity cDNA Reverse Transcriptase kit. Quantitative PCRs used a TaqMan 2× Enzyme Master Mix, TaqMan probes, and custom primers from Invitrogen. Following 44 cycles in an ABI Prism® 7000 Sequence Detection System (Applied Biosystems), cycle threshold (Ct) values were adjusted such that the highest wild-type value averaged to 100.

Bioinformatics and Statistical Analyses—Multiple sequence alignment of protein sequences of the 33 human tetraspanins was conducted using Clustal Omega (39). Statistical analyses were carried out using a one-way analysis of variance followed by Dunnett’s test.

RESULTS

ADAM10 Associates with Tetraspanins in Platelets—The ectodomain sheddase ADAM10 has been reported to associate with tetraspanins in a variety of cell lines (23, 24). The platelet was initially selected as a good model for a human cell line that expresses ADAM10 and multiple tetraspanins. Thus, to determine whether ADAM10 interacts with tetraspanins on human platelets, they were surface-biotinylated and subjected to lysis in two different detergents: 1% Brij97, which largely retains tetraspanin-tetraspanin interactions, and the more stringent 1% Triton X-100, which disrupts tetraspanin-tetraspanin interactions (12). Following immunoprecipitation of ADAM10 or control proteins, co-immunoprecipitated surface proteins were detected by streptavidin Western blotting. Under Brij97 lysis, ADAM10 was found to co-immunoprecipitate a pattern of proteins strikingly similar to CD151 (Fig. 1), which was chosen because it is the best characterized platelet tetraspanin (21).
FIGURE 2. Tspan14, Tspan15, and Tspan33 interact with and promote the maturation of ADAM10. A–C, HEK-293T cells were transfected with FLAG-tagged mouse forms of a panel of 10 tetraspanins previously found to be expressed by human platelets (26, 29) in the absence (–) or presence (+) of HA-tagged bovine ADAM10. The cells were surface-cross-linked with DTSSP, lysed in 1% Triton X-100, and subjected to anti-FLAG immunoprecipitation (IP) in 1% Triton X-100 containing 0.1% SDS. A, the immunoprecipitates were reduced to break the cross-linker, and ADAM10 was detected by anti-HA Western blotting. The immature (upper band) and mature (lower band) forms of ADAM10 were detected and quantified using the Odyssey Infrared Imaging System from LI-COR Biosciences, and a graph is presented to show the mean total ADAM10 with S.E. from three experiments (**, p < 0.01 compared with all other tetraspanins). B, ADAM10 in whole cell lysates was similarly analyzed by anti-HA Western blotting, and a graph is presented to show the percentage of mature ADAM10 for each transfection (n = 3; **, p < 0.01 compared with ADAM10 only; error bars represent S.E.). C, expression of tetraspanins was confirmed by anti-FLAG Western blotting. The tetraspanins varied in their expression levels; Tspan2 was not readily detected in this figure but was detectable on longer exposures (data not shown). D–F, palmitoylation of Tspan14, Tspan15, and Tspan33 is not essential for ADAM10 interaction or promotion of maturation. The experiment was performed as described in A–C except that wild-type (WT) and palmitoylation mutant (PM) forms of human tetraspanins were compared, and the experiment was performed in 1% digitonin lysis buffer in the absence of DTSSP cross-linking (data are representative of three experiments; **, p < 0.01).

adopted using FLAG epitope-tagged tetraspanins and HA-tagged ADAM10. This was followed by surface chemical cross-linking with DTSSP with the aim of capturing direct tetraspanin-ADAM10 associations and co-immunoprecipitation under highly stringent lysis conditions (1% Triton, 0.1% SDS) to retain only cross-linked interactions. Importantly, DTSSP contains a disulfide bond that can be broken by reducing agents, thus allowing individual proteins to be detected by Western blotting at their appropriate molecular weights. This surface chemical cross-linking protocol has the added benefit of restricting the analysis to surface proteins, therefore avoiding intracellular aggregated proteins that can be a problem in transient transfection of HEK-293T cells. Following immunoprecipitation of the 10 tetraspanins, Tspan14, Tspan15, and Tspan33 shared the capacity to immunoprecipitate significantly more surface ADAM10 than the others (Fig. 2A). These three tetraspanins each interacted preferentially with the low molecular weight form of ADAM10, which corresponds to mature ADAM10 with a cleaved prodomain. In addition, Western blotting of whole cell lysates showed that Tspan14, Tspan15, and Tspan33 each promoted significant maturation of ADAM10 (Fig. 2B). Finally, expression of each tetraspanin was confirmed, albeit at levels that varied from relatively strong for CD9, Tspan14, Tspan15, and Tspan33 to relatively weak for Tspan2, which was only detectable upon longer exposures (Fig. 2C and data not shown). Taken together, these data suggest that among platelet tetraspanins Tspan14, Tspan15, and Tspan33 interact with ADAM10 and promote its maturation. This is the first reported function for Tspan14 or Tspan15; a gene knockout study has demonstrated an essential role for Tspan33 in mouse erythropoiesis (22).

Tetraspanin Palmitoylation Is Not Required for Tspan14, Tspan15, and Tspan33 to Promote ADAM10 Maturation—Palmitoylation of tetraspanins on multiple cysteine residues at their transmembrane-cytoplasmic junctions is known to promote tetraspanin-tetraspanin interaction and the formation of tetraspanin microdomains. Indeed, mutation of these cysteines impairs tetraspanin-tetraspanin interactions (12). If such mutants of Tspan14, Tspan15, and Tspan33 retained the capacity to regulate ADAM10, this would provide additional evidence in favor of a direct interaction (that is, an interaction that is not due to chemical cross-linking of a complex of proteins containing transfected proteins and endogenous tetraspanins that act as linkers). Interestingly, the previously reported regulation of ADAM10 by Tspan12 was dependent on palmitoylation of the latter (24), raising the possibility that the observed interaction was indirect. In an experiment with the same methodology as that shown previously in Fig. 2, A–C, palmitoylation mutants of Tspan14, Tspan15, and Tspan33 retained the capac-
ity to interact with surface ADAM10 and to promote its maturation (supplemental Fig. II).

To provide further evidence in support of these observations, co-immunoprecipitations were performed from non-cross-linked cells lysed in 1% digitonin. This is a detergent that precipitates cholesterol and large tetraspanin complexes but in which smaller tetraspanin-partner interactions appear to remain soluble (38). Indeed, digitonin has previously proved useful in identifying tetraspanin-partner interactions (12). Under digitonin lysis conditions, Tspan14, Tspan15, and Tspan33 co-immunoprecipitated robustly with ADAM10 in a manner that did not require palmitoylation of the tetraspanins (Fig. 2D). Promotion of ADAM10 maturation also did not require palmitoylation (Fig. 2E). As controls, mutation of palmitoylation sites did not affect tetraspanin expression (Fig. 2F), and CD9 failed to co-immunoprecipitate with ADAM10 or promote its maturation (Fig. 2, D–F). Taken together, these data are consistent with a direct interaction between ADAM10 and the tetraspanins Tspan14, Tspan15, and Tspan33.

Tspan14, Tspan15, and Tspan33 Are Members of the TspanC8 Subgroup of Tetraspanins—Tspan14, Tspan15, and Tspan33 share a relatively high protein sequence identity of between 31 and 39% in human and belong to a subgroup of six related tetraspanins that includes the functionally uncharacterized Tspan5, Tspan10, and Tspan17 (Fig. 3A). These six tetraspanins have eight cysteine residues within their main extracellular region unlike other tetraspanins that have four, six, or seven. These cysteines appear to be structurally important through the formation of disulfide bonds (12). Hence, we have termed these six tetraspanins the TspanC8 subgroup.

To determine whether all six TspanC8 tetraspanins share the capacity to interact with and promote ADAM10 maturation, ADAM10 was co-transfected into HEK-293T cells with each TspanC8 tetraspanin, and ADAM10 co-immunoprecipitation and maturation following digitonin lysis were detected by Western blotting as described in Fig. 2, A–C. The TspanC8 tetraspanins each interacted with ADAM10, but control tetraspanins CD9, CD151, and Tspan9 did not (Fig. 3B). Among the TspanC8 tetraspanins, Tspan10 interacted with ADAM10 relatively poorly (Fig. 3B), which was consistently observed in three experiments (data not shown). Nevertheless, each TspanC8 tetraspanin promoted significant ADAM10 maturation unlike the non-TspanC8 tetraspanin controls (Fig. 3C), and expression of the tetraspanins was confirmed in Fig. 3D. These data suggest that the six TspanC8 tetraspanins share a common function in regulating ADAM10.

Tspan12 is not a TspanC8 tetraspanin (Fig. 3A) but was reported previously to promote ADAM10 maturation (24). However, this study did not include any TspanC8 tetraspanins that would have allowed their comparison with Tspan12. To address whether TspanC8 tetraspanins and Tspan12 interact with ADAM10 and promote maturation in a comparable manner, interaction and maturation were measured by co-immunoprecipitation in digitonin lysis buffer. In these experiments,
TspanC8 Subgroup Regulates ADAM10 Maturation and Expression

The amount of transfected FLAG-Tspan14 was titrated down to allow comparison with the relatively poorly expressed FLAG-Tspan12. Unlike Tspan14, Tspan12 did not interact with ADAM10 (Fig. 4A) or induce ADAM10 maturation (Fig. 4B) despite similar expression levels at the lower end of the Tspan14 titration (Fig. 4C). In a complimentary set of experiments, GFP-tagged tetrascansins were used because GFP-Tspan14, and the DTSSP cross-linking method was used as described previously in Fig. 2, A–C (supplemental Fig. III). Consistent with FLAG-Tspan12 in Fig. 4, GFP-Tspan12 failed to interact with ADAM10 or promote its maturation (supplemental Fig. III). Taken together with the previously published data, this suggests that although Tspan12 can induce ADAM10 maturation under some circumstances the TspanC8 tetrascansins may be the major tetrascanin regulators of ADAM10.

Tspan14 Is the Major TspanC8 Tetrascanin in HUVECs and Is Essential for Normal ADAM10 Surface Expression—Because overexpression of TspanC8 tetrascansins promotes ADAM10 maturation, a process that occurs during ADAM10 trafficking to the plasma membrane, it is possible that TspanC8 tetrascansins are required for such trafficking. This would be comparable with the way in which other tetrascansins are known to promote trafficking of other partners (12, 40). To test this theory, siRNA knockdown of TspanC8 tetrascansins was carried out followed by flow cytometry to detect surface ADAM10 expression. Because siRNA knockdown is not possible in platelets, primary HUVECs were selected because of the importance of ADAM10 in endothelial cell function (1). To initially determine the relative expression levels of TspanC8 tetrascansins in HUVECs, RT-PCR was carried out because effective antibodies are not available to any TspanC8 tetrascanin. Tspan14 mRNA was found to be more abundant than the rest of the TspanC8 tetrascansins combined (Fig. 5A). These data were comparable with next generation sequencing of HUVECs that yielded the following number of mRNA transcripts per million: 9.1 for Tspan5, 0.1 for Tspan10, 56.9 for Tspan14, 14.6 for Tspan15, 24.7 for Tspan17, and 0 for Tspan33. Tspan14 knockdown with two different siRNA duplexes was found to decrease Tspan14 mRNA by over 75% as detected by RT-PCR (Fig. 5B). More importantly, each significantly reduced surface ADAM10 by over 50% (Fig. 5, C and D). It is possible that residual Tspan14 and other TspanC8 tetrascansins in these cells is the reason why the reduction in surface ADAM10 was not complete.

To determine the effect of Tspan14 knockdown on ADAM10 activity, HUVEC lysates were Western blotted for the ADAM10 substrate VE-cadherin (10). Tspan14 knockdown significantly reduced VE-cadherin cleavage in line with the reduction in ADAM10 expression level (Fig. 5, E and F). As an additional control, VE-cadherin cleavage was further reduced by ADAM10 knockdown (Fig. 5, E and F), and the level of ADAM10 protein knockdown was almost 100% as detected by flow cytometry (Fig. 5C). In combination with the flow cytometry data, these ADAM10 activity data are consistent with the idea that TspanC8 tetrascansins are essential for normal ADAM10 surface expression.

ADAM10 Stability in the A549 Cell Line Is Not Reduced following Tspan14 Knockdown—The reduced ADAM10 surface expression following Tspan14 knockdown could be due to impaired trafficking to the cell surface and/or reduced stability. To address the latter possibility, a cell surface biotinylation assay was used in Tspan14 knockdown A549 human lung epithelial cells. This cell line was chosen for these experiments because its TspanC8 tetrascanin profile is similar to that of HUVECs (Fig. 6A). In addition, A549 has the advantage of being a faster growing cell line that is more amenable to biochemical analyses than primary HUVECs. Similar to HUVECs, Tspan14 knockdown significantly reduced the level of surface ADAM10, which in this case was detected by surface biotinylation and ADAM10 immunoprecipitation followed by neuvidin Western blotting (Fig. 6, B and C). However, there was no evidence for increased ADAM10 degradation following Tspan14 knockdown relative to negative control siRNA over 24, 36, and 48 h postbiotinylation (Fig. 6B). Instead, quantitation revealed that loss of biotinylated ADAM10 over time appeared slightly slower following Tspan14 knockdown (Fig. 6C). These data suggest that impaired ADAM10 trafficking to the cell surface is the main consequence of Tspan14 knockdown.

Tspan33 Is the Major TspanC8 Tetrascanin in the Erythrocyte Lineage and Is Essential for Normal ADAM10 Expression—To determine whether TspanC8 tetrascansins regulate ADAM10 in vivo, ADAM10 expression was investigated in cells from the Tspan33-deficient mouse, which is the only currently available knock-out mouse for a TspanC8 tetrascanin. The two major blood cell types, platelets and erythrocytes, were selected as the subjects of this analysis because their relative Tspan33 expression levels were likely to be quite distinct; Tspan33 mRNA is expressed by mouse erythrocytes (22) but is

3 J. M. Herbert and R. Bicknell, unpublished data.
minimal in mouse platelets (supplemental Fig. I). To test this hypothesis, TspanC8 tetraspanin mRNA levels were measured by RT-PCR in mouse megakaryocytes and erythroid progenitors because mature platelets and erythrocytes carry relatively small amounts of mRNA. Tspan33 expression in mouse megakaryocytes was almost undetectable in contrast to the relatively high expression of Tspan14 and weaker expression of Tspan5 and Tspan17 (Fig. 7A). Conversely, Tspan33 was the major TspanC8 tetraspanin in erythroid progenitors compared with almost undetectable levels of Tspan5 and Tspan14 (Fig. 7B). No compensatory up-regulation of other TspanC8 tetraspanins was apparent in Tspan33-deficient cells (data not shown). As a control, primer/probe efficiencies were found to be similar for each TspanC8 tetraspanin (supplemental Fig. IV).

Consistent with the extremely low relative level of Tspan33 in megakaryocytes, ADAM10 surface expression was normal on Tspan33-deficient mouse platelets (Fig. 7C). In contrast, Tspan33-deficient erythrocytes exhibited an approximate 90% reduction in surface ADAM10 (Fig. 7D), which was consistent with similarly reduced whole cell ADAM10 expression as determined by Western blotting (Fig. 7E). Importantly, defective ADAM10 protein expression was not due to any reduction in ADAM10 mRNA in the erythroid lineage (Fig. 7F). Together these data demonstrate that TspanC8 tetraspanins regulate ADAM10 in vivo. The distinct repertoires of TspanC8 tetraspanins in different cell types may allow ADAM10 targeting to specific substrates and may ultimately be exploited in therapies that target specific TspanC8-ADAM10 complexes.
DISCUSSION

The metalloprotease ADAM10 cleaves over 40 transmembrane targets, including the platelet-activating collagen receptor glycoprotein VI (7, 8). Because ADAM10 is compartmentalized into tetraspanin membranes on various cell types (23, 24), including platelets (Fig. 1), the present study began with the hypothesis that one or more platelet tetraspanins would specifically associate with and regulate ADAM10. This hypothesis was proved correct by the discovery that three platelet tetraspanins could interact with and promote ADAM10 maturation. These were Tspan14, Tspan15, and Tspan33, the former two of which were previously uncharacterized. Because of their relatively strong sequence identities, including eight cysteine residues within their major extracellular region, they were named the TspanC8 subgroup of tetraspanins. This subgroup contains three other uncharacterized tetraspanins, Tspan5, Tspan10, and Tspan17, which each were able to interact with and promote ADAM10 maturation. Furthermore, analyses of the only available knock-out mouse for a TspanC8 tetraspanin (Tspan33) and tetraspanin knockdown in cultured primary endothelial cells demonstrated an essential role for TspanC8 tetraspanins in ADAM10 trafficking to the cell surface. Reduced endothelial cell surface ADAM10 correlated with reduced activity toward its substrate (VE-cadherin). This study has therefore defined a function for a largely uncharacterized subgroup of tetraspanins and has identified a novel global regulatory mechanism for ADAM10.

The hypothesis behind this work was conceived following two previous reports that ADAM10 was tetraspanin-associated in leukocyte, breast cancer, and neuroblastoma cell lines (23, 24). Moreover, Tspan12 was proposed as an ADAM10-interacting tetraspanin (24). However, because ADAM10 appears to be ubiquitous but Tspan12 is not (16), it seemed likely that other tetraspanins would interact with ADAM10. Analyses of the Tspan12-deficient mouse did not address a potential role in ADAM10 regulation but instead demonstrated a role for Tspan12 in the regulation of Frizzled-4-induced β-catenin signaling (16). Tspan12 deficiency leads to defective vascular development in the retina, and Tspan12 mutations cause the human blinding disease familial exudative vitreoretinopathy, thus phenocopying mutations in the Frizzled-4 signaling pathway (17, 18). To address whether Tspan12 could regulate ADAM10 in a manner comparable with that of TspanC8 tetraspanins, Tspan12 was compared with Tspan14 for its capacity to interact with and promote ADAM10 maturation. Using two different transiently transfected Tspan12 constructs (FLAG- and GFP-tagged) in two different types of co-immunoprecipitation experiment (digitonin lysis or DTSSP surface cross-linking), Tspan12 appeared unable to interact with ADAM10 and promote its maturation. How can this finding be resolved with the previous, apparently contradictory publication (24)? It is possible that this earlier finding was a consequence of up-regulation of TspanC8 expression at the transcriptional level due to the stable Tspan12 transfection used in the study (24). Tspan12 transfection can certainly promote Frizzled-4-induced β-catenin signaling in a cell line model (16), and this could in theory induce the transcription of one or more TspanC8 tetraspanins. This could facilitate indirect Tspan12 interaction with ADAM10 and maturation of the latter (24). A candidate is Tspan5, which was identified as a β-catenin target gene in a microarray screen of colorectal cancer cells (41). Consistent with the theory that the previously observed Tspan12-ADAM10 interaction could be bridged by a TspanC8 tetraspanin, the interaction was only detected in relatively non-stringent lysis...
conditions (0.5% Brij97) (24) that would maintain tetraspanin-tetraspanin interactions (12). Indeed, the interaction and ADAM10 maturation were lost upon mutation of the Tspan12 palmitoylation sites (24), which are required for normal tetraspanin-tetraspanin association (12). Importantly, mutation of palmitoylation sites in the TspanC8 tetraspanins did not abolish maturation of ADAM10 or significantly inhibit the interaction in the present study, suggesting that these are bona fide ADAM10-interacting tetraspanins.

The key finding that TspanC8 tetraspanin deficiency results in reduced ADAM10 cell surface expression could potentially be due to impaired trafficking to the plasma membrane and/or reduced ADAM10 stability. Reduced stability is unlikely to be involved because knockdown of Tspan14, the major TspanC8
TspanC8 Subgroup Regulates ADAM10 Maturation and Expression

tetraspanin in the A549 epithelial cell line, failed to show any increased ADAM10 degradation over time using a cell surface biotinylation approach. Importantly, ADAM10 surface expression was significantly reduced by Tspan14 knockdown at the beginning of the experiment. Therefore, TspanC8 tetraspanins are likely to promote ADAM10 trafficking to the cell surface. Promotion of partner trafficking is emerging as a general concept in tetraspanin biology. For example, tetraspanin CD81 is essential for normal surface trafficking of CD19, a key component of B cell receptor signaling, such that mutation of CD81 in humans results in a loss of surface CD19 and profound immunodeficiency (40). An important question that arises from the current study concerns the mechanism by which the TspanC8 tetraspanins promote trafficking of ADAM10 to the cell surface. ADAM10 was recently shown to possess an arginine-based endoplasmic reticulum (ER) retention motif that prevents efficient trafficking of the metalloprotease to the cell surface (42). However, it was not clear from that study how the retention motif is overcome and ADAM10 is released from the ER. One theory in the study of such arginine-based ER retention motifs is that they can be masked upon successful interaction with a partner protein, which then allows the correctly assembled complex to be transported from the ER (43). It is therefore possible that ADAM10 is retained in the ER until interaction with a TspanC8 tetraspanin, which masks the retention motif.

A striking finding from the present study was that as many as six tetraspanins could regulate ADAM10. Such functional compensation among tetraspanins is not without precedent albeit on a smaller scale. The clearest example is for the related tetraspanins CD9 and CD81, which share 44% protein sequence identity in human and share some common partners, namely the immunoglobulin superfamily proteins EWI-2 and EWI-F (CD9P-1) (12). CD81 was shown to partially compensate for the fertilization defect observed in CD9-deficient mice (12). In addition, a role for these tetraspanins in the maintenance of normal lung function was only revealed by the observation of a chronic obstructive pulmonary disease-like phenotype in the double knock-out mouse but not in either of the single knockouts (44). Compensation between TspanC8 tetraspanins is likely to complicate future analyses of knockdown cells and knock-out animals. An additional problem is the lack of effective antibodies to any TspanC8 tetraspanin, which leaves RT-PCR as the current best option for quantifying mRNA expression levels in different cell types. This technique was used in the present study to demonstrate that Tspan33 and Tspan14 are the major TspanC8 tetraspanins in mouse erythroid cells and HUVECs, respectively. This explains the striking reductions in surface ADAM10 on erythrocytes and HUVECs deficient for these tetraspanins. The Tspan33-deficient mice used in this study exhibit impaired erythropoiesis (22), but it is currently unclear whether an ~90% reduction in surface ADAM10 could be responsible for this phenotype. Finally, ADAM10 was found to be normally expressed on the surface of platelets from these mice, which was consistent with the almost undetectable level of Tspan33 mRNA in their megakaryocyte precursors. Because Tspan14 is the major TspanC8 tetraspanin in this lineage, defective platelet ADAM10 expression in a Tspan14 knock-out mouse would be predicted.

ADAM10 has considerable therapeutic potential due to the important roles of its many target proteins in inflammation, thrombosis, cancer, and Alzheimer disease (1, 4). Despite its potential in therapy, all clinical trials involving ADAM10 have so far failed due to problems with toxicity (4). However, this study has identified the TspanC8 tetraspanins as the regulators of ADAM10 maturation and trafficking to the cell surface. The ADAM10 interaction with six TspanC8 tetraspanins may have evolved to allow targeting of ADAM10 to specific substrates or compartments within the cell. Targeting specific TspanC8 tetraspanin–ADAM10 complexes raises the possibility of targeting the metalloprotease in a cell type- or substrate-specific manner. Such an approach could target specific disease processes such as cancer, Alzheimer disease, and cardiovascular disease while minimizing problems with toxicity.

Acknowledgments—We thank Eric Rubinstein for reagents and discussions, Victoria Heath for comments on the manuscript, Alexandra Mazharian for megakaryocyte advice, the Biomedical Services Unit for housing mice, and Phil Stone for providing HUVECs. We are also grateful to John Herbert and Roy Bicknell for sharing unpublished data.

Addendum—Prox et al. (45) have recently identified the TspanC8 tetraspanin Tspan15 in a screen for ADAM10-interacting proteins. Consistent with our data on Tspan14, Tspan33, and other TspanC8 tetraspanins, Tspan15 was found to promote ADAM10 exit from the endoplasmic reticulum, maturation, cell surface expression, and activity (45).

REFERENCES
TspanC8 Subgroup Regulates ADAM10 Maturation and Expression


eromultimeric membrane proteins. EMBO Rep. 6, 717–722