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Staphylococcus aureus protein A activates TACE through EGFR-dependent signaling

Marisa I Gómez[1], Maghnus O Seaghdha[2] and Alice S Prince[1,*]

[1]Department of Pediatrics and Pharmacology, College of Physicians & Surgeons, Columbia University, New York, NY, USA and [2]Department of Microbiology, Moyne Institute of Preventative Medicine, Trinity College, Dublin, Ireland

Among the many adhesins and toxins expressed by *Staphylococcus aureus*, protein A is an exceptionally complex virulence factor, known to interact with multiple eukaryotic targets, particularly those with immunological functions. Protein A acts as a ligand that can mimic TNF-α to activate TNFR1 and subsequent proinflammatory signaling. It also stimulates the cleavage of TNFR1 from the surface of epithelial cells and macrophages, which serves to limit TNF-α signaling. We characterized the signaling pathway responsible for TNFR1 shedding and identified protein A mutants which could activate TNFR1-dependent signaling, but were unable to activate TACE, the TNFR1 sheddase. Activation of TACE was dependent upon a discrete interaction between the previously defined IgG-binding domain of protein A and the epidermal growth factor receptor (EGFR), which in turn induced TACE phosphorylation through a c-Src-erk1/2-mediated cascade. This novel interaction was independent of the autocrine activation of EGFR and protein A-induced TGF-α was neither required nor sufficient to activate TNFR1 shedding. Thus, staphylococci exploit the ubiquitous and multifunctional EGFR to regulate the availability of TNFR1 on mucosal and immune cells.

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Introduction

Staphylococcus aureus is a common human pathogen capable of causing a wide range of infection in normal and compromised hosts (Lowy, 1998). It is characterized by an exceptional ability to exploit host immune functions (Foster, 2005), and several clinically important interactions are mediated by protein A, a surface virulence factor that is highly conserved and abundantly expressed in the lung (Lowy, 1998; Goerke *et al*, 2000). Protein A can impede phagocytosis by binding the Fc component of immunoglobulin (Uhlen *et al*, 1984; Foster, 2005), activate clotting by binding von Willebrand factor (Hartleib *et al*, 2000), act as a superantigen for B cells by binding the Fab region of VH3 bearing IgM (Moks *et al*, 1986; Sasso *et al*, 1989; Roben *et al*, 1995; Jansson *et al*, 1998) and through its activation of TNFR1, initiate staphylococcal pneumonia (Gómez *et al*, 2004).

Activation and regulation of the TNF-α signaling cascade is critically important in the host response to infection (Chen and Goeddel, 2002; Aggarwal, 2003; Saunders *et al*, 2005; Rahman and McFadden, 2006). Whereas the production of TNF-α is predominantly by immune cells, mucosal epithelial cells express TNFR1 and are highly responsive to local concentrations of TNF-α. During staphylococcal pneumonia, TNFR1 is specifically mobilized to the apical surface of the airway epithelium, providing access to inhaled staphylococci (Gómez *et al*, 2004). The abundance of TNFR1 is controlled by mobilization from intracellular stores and cleavage from the cell surface (Bradley *et al*, 1995; Peschon *et al*, 1998; Jones *et al*, 1999; Reddy *et al*, 2000; Wang *et al*, 2003a; Xanthoulea *et al*, 2004; Garton *et al*, 2006). Release of the TNFR1 ectodomain prevents ongoing signaling and serves to neutralize free TNF-α in the airway.

The interactions of protein A and TNFR1 are essential for the pathogenesis of staphylococcal pneumonia. TNFR1-null mice have decreased rates of infection as protein A-defective mutants of *S. aureus* did in wild-type animals (Gómez *et al*, 2004). TNFR1 recognition is mediated by the same domain of protein A that binds IgG (Gómez *et al*, 2006) and mimics TNF-α proinflammatory signaling (Gómez *et al*, 2004). In addition, we noted that protein A also induces the cleavage of TNFR1 and its shedding from the epithelial surface. Cleavage of TNFR1 is mediated by TACE, the TNF-α converting enzyme, a metalloprotease of the ADAM family that is a central regulator of TNF-α signaling (Peschon *et al*, 1998; Reddy *et al*, 2000). Such metalloproteases are important in many aspects of lung defense, carcinogenesis and repair (Peschon *et al*, 1998; Reddy *et al*, 2000; Garton *et al*, 2003; Mezyk *et al*, 2003; Tsakadze *et al*, 2006). However, bacterial activation of TACE has not been previously characterized. As protein A induces TNFR1 shedding, we postulated that this virulence factor must activate TACE, and in the experiments detailed, outline the pathway through which this occurs.

Results

S. aureus protein A-induces TNFR1 mobilization and receptor shedding

Protein A rapidly induced recruitment of TNFR1 to the cell surface (Figure 1A) and sTNFR1 was detected as soon as 2 h after epithelial stimulation and continued to increase for at least 24 h (Figure 1B). As sTNFR1 accumulated in the culture supernatant, the total amount of cell-associated TNFR1

*Corresponding author. Department of Pediatrics and Pharmacology, College of Physicians & Surgeons, Columbia University, Black Building BB4-416, 650 West 168th Street, New York, NY 10032, USA.
Tel.: +1 212 305 4193; fax: +1 212 342 5728;
E-mail: asp7@columbia.edu

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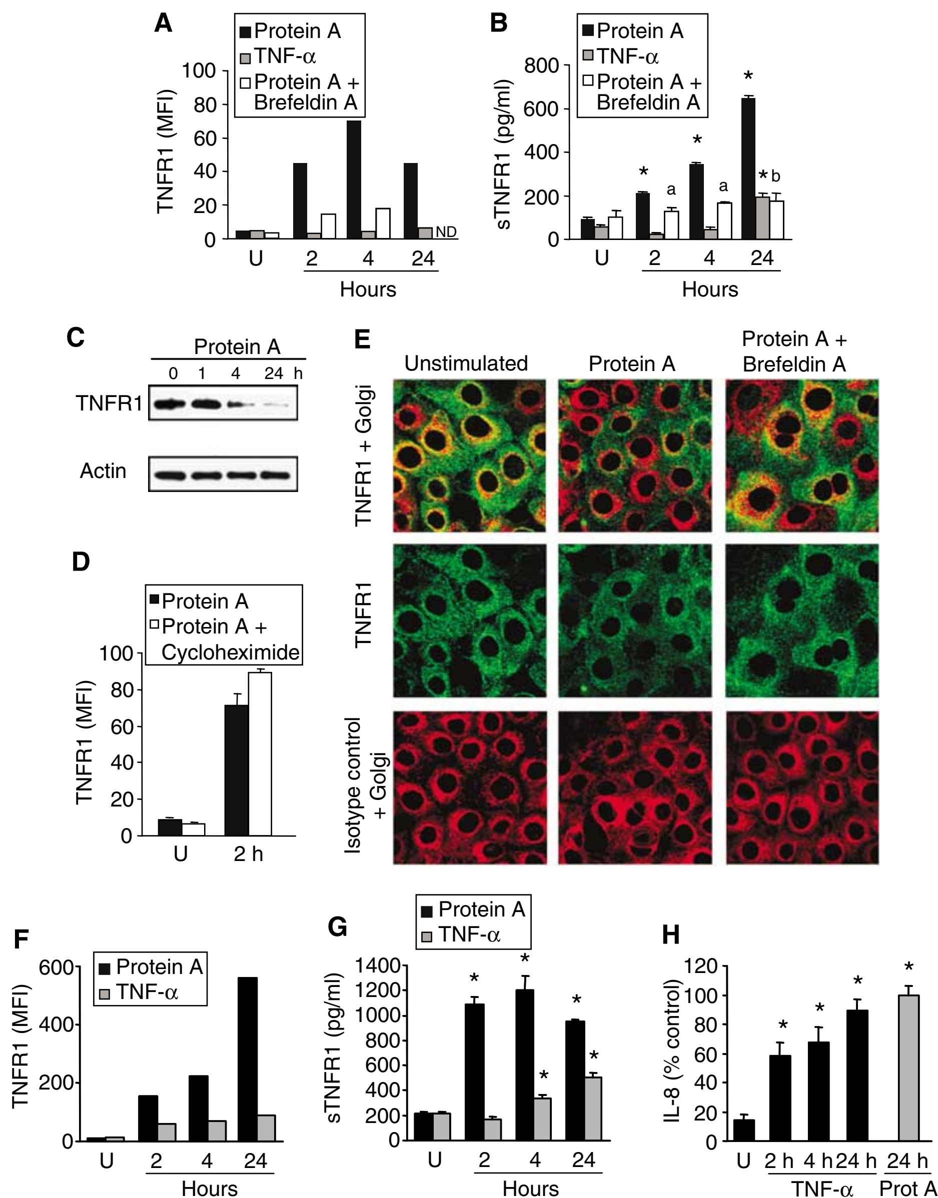

Figure 1 TNFR1 mobilization and shedding. 1HAEo− (**A, B, D, H**) and Raw (**F, G**) cells were stimulated with protein A, TNF-α or media alone (U: unstimulated). 1HAEo− cells were also stimulated with protein A in the presence of Brefeldin A (A, B) or cycloheximide (D). At different time points after stimulation, cells were stained for surface expression of TNFR1 (A, D, F) and soluble TNFR1 (sTNFR1) (B, G) or IL-8 (**H**) were measure by ELISA. MFI: mean fluorescence intensity. One representative experiment of three is shown. (B, G, H) Data represent the mean and s.d. of six wells and in (H) are represented as percentage of IL-8 induced by protein A. (*$P<0.05$, compared to unstimulated, $^{a}P<0.05$, $^{b}P<0.01$, both compared to Protein A alone, Student's *t* Test). (**C**) 1HAEo− whole cell lysates were immunoblotted for TNFR1 and actin. (**E**) 1HAEo− cells grown on transwells were incubated with the Golgi probe BODIPY TR (red), stimulated with protein A in the presence or absence of Brefeldin A and stained for TNFR1 (green). Colocalization of TNFR1 and Golgi apparatus appears yellow. *x*–*y* scans are shown.

decreased (Figure 1C). TNFR1 shedding was dependent upon receptor mobilization from Golgi stores as Brefeldin A prevented both TNFR1 mobilization and shedding in Protein A-stimulated cells (Figure 1A and B). In contrast, cycloheximide treatment did not have any significant effect on TNFR1 mobilization (Figure 1D) or shedding even at later time points (data not shown). TNFR1 transcription was not affected by protein A stimulation (data not shown). TNFR1 mobilization from Golgi stores in response to protein A was confirmed by confocal imaging. TNFR1 colocalized with a Golgi probe in the unstimulated but not in the Protein A stimulated cells (Figure 1E). The natural TNFR1 ligand, TNF-α, did not induce mobilization of TNFR1 (Figure 1A) or stimulate receptor shedding (Figure 1B). A small amount of sTNFR1 was detectable 24 h after TNF-α stimulation, equivalent to 30% of the response induced by protein A.

To determine whether TNFR1 shedding is limited to the airway epithelial cells, the effects of protein A on superficial TNFR1 on macrophages was examined. Whereas TNFR1 is abundant on the macrophage surface, additional receptor was mobilized and shed in response to protein A but not TNF-α (Figure 1F and G). By 24 h the amount of shed TNFR1 in response to TNF-α was equivalent to only 53% of that induced by protein A (Figure 1G). To verify that

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basal levels of TNFR1 on the cell surface of unstimulated airway epithelial cells are sufficient to mediate TNF-α signaling, IL-8 production in response to TNF-α was determined and found to be comparable to that induced by protein A (Figure 1H).

Distribution of TACE and TNFR1 on the surface of airway epithelial cells

TACE is the major metalloprotease that cleaves TNFR1 from the surface of airway epithelial cells in response to protein A, as demonstrated by RNA interference (Figure 2A). Depletion of TACE expression significantly reduced protein A-induced TNFR1 shedding. For TACE to efficiently cleave TNFR1, both the protease and its substrate must be available on the apical surface of the airway cell. TACE is abundant on the surface of the airway epithelial cells (Figure 2B). As TACE transcription is not inducible by protein A (data not shown); nor is the distribution of TACE changed following cell stimulation (Figure 2C), we postulated that the substrate TNFR1 must be actively mobilized to the cell surface. Using confocal imaging we found that TNFR1, which is mobilized in response to protein A stimulation (Figure 1A), colocalizes with TACE on the apical surface of the cells (Figure 2D). This finding was confirmed using human airway epithelial cells in primary culture (Figure 2D).

Protein A induces TACE phosphorylation

Previous reports suggest that metalloproteinases are activated by reactive oxygen species (ROS) generated in response to bacterial infection (Hino *et al*, 1999; Fang, 2004). However, protein A activation of TACE was not inhibited by the ROS scavenger *n*-propyl gallete (data not shown), suggesting that other pathways are involved. TACE is phosphorylated at both ser 819 and thr 735 by erk1/2 MAPK in transfected cell lines stimulated with PMA or growth factors (Diaz-Rodriguez *et al*, 2002; Fan *et al*, 2003). The airway epithelial cells express the pro-form and the mature form (devoid of the pro-domain) of TACE as it has been described for other cell lines. We detected threonine phosphorylation of the mature form of TACE as early as 5–10 min and maximal amounts at 30 min following protein A stimulation (Figure 3A). TACE phosphorylation persisted for up to 24 h (Figure 3B). Phosphorylation was not observed in cells treated with the MEK inhibitor UO126 (Figure 3A). No serine phosphorylation of TACE was detected (data not shown). Association of phosphorylated erk1/2 with the mature form of TACE was detected at 5 min following

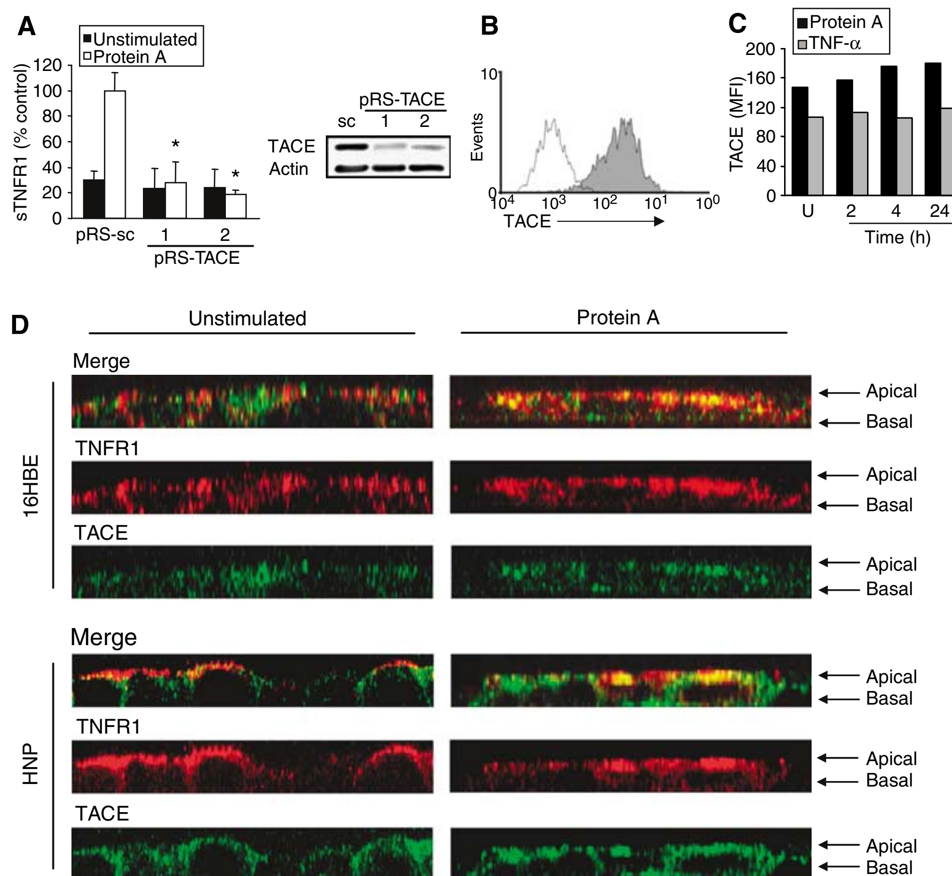

Figure 2 TNFR1-TACE colocalization in airway epithelial cells. (**A**) 16HBE cells expressing pRS-TACE or pRS-sc were stimulated with protein A and sTNFR1 was determined by ELISA. Data represent the mean and s.d. of sextuplicate wells and are represented as percentage of sTNFR1 detected in cells expressing pRS-sc after protein A stimulation. (*$P<0.05$ compared to Protein A stimulation in pRS-sc cells, Student's *t* Test). Whole cell lysates from 16HBE cells expressing pRS-sc or pRS-TACE immunoblotted for TACE and actin are shown as control. (**B**) TACE expression was determined by flow cytometry. Solid histogram: cells stained with antibody to TACE; open histogram: cells stained with normal goat serum as control. (**C**) 1HAEo− cells were stimulated with protein A, TNF-α or media alone (U: unstimulated) and stained for surface expression of TACE. MFI: mean fluorescence intensity. One representative experiment of three is shown. (**D**) Polarized 16HBE or primary human nasal polyp (HNP) cells grown on transwells and stimulated with protein A were stained for TACE (green) and TNFR1 (red) and analyzed by confocal imaging. Colocalization of TACE and TNFR1 appears yellow. *Z*-sections are shown.

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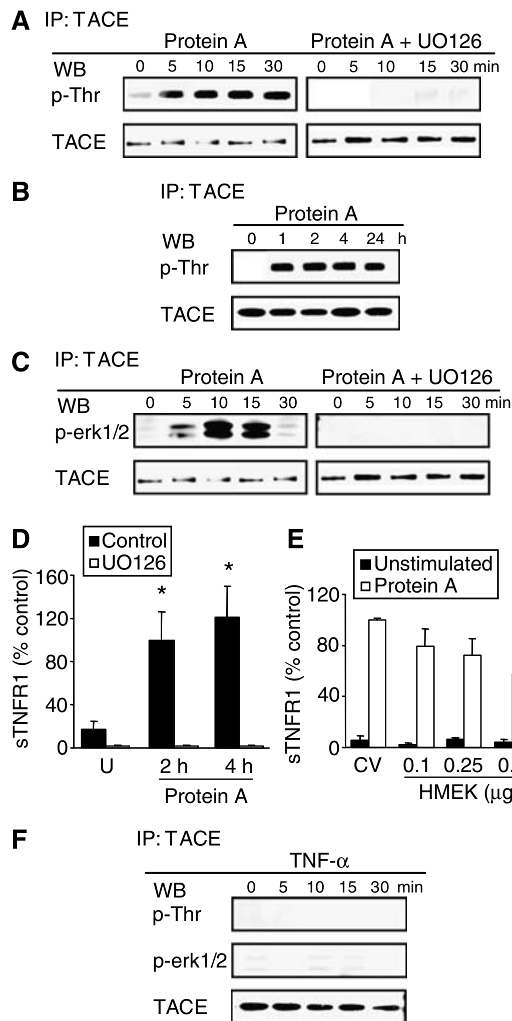

Figure 3 Erk1/2-dependent TACE phosphorylation. (**A–C**) TACE immunoprecipitates (IP) from cell lysates stimulated with protein A in the presence or absence of the MEK inhibitor UO126 were immunoblotted (WB) for phospho-threonine (p-Thr) (A, B), phospho-erk1/2 (p-erk1/2) and erk1/2 (C) as well as TACE as loading control. (**D**) 1HAEo− cells were stimulated with protein A in the presence or absence of the MEK inhibitor UO126 and soluble TNFR1 (sTNFR1) was measured by ELISA. Data represent the mean and s.d. of six wells and are expressed as percentage of the sTNFR1 detected after stimulation with protein A for 2 h (*$P<0.01$, Student's *t* Test). (**E**) 1HAEo− cells were transiently transfected with a MEK dominant-negative mutant (HMEK) or vector control (CV) and sTNFR1 was measured by ELISA. Data represent the mean and standard deviation of 4 wells and is presented as percentage of the sTNFR1 detected in CV transfected cells after protein A stimulation (*$P<0.01$, Student's *t* Test). (**F**) TACE IP from cell lysates stimulated with TNF-α were immunoblotted (WB) for phospho-threonine (p-Thr) and phospho-erk1/2 (p-erk1/2), as well as TACE as loading control.

protein A stimulation (Figure 3C), whereas phospho-erk1/2, in complex with TACE, was not present in cells treated with the MEK inhibitor (Figure 3C). When erk1/2 phosphorylation was prevented with the MEK inhibitor UO126, airway epithelial cells did not release sTNFR1 in response to protein A (Figure 3D). Expression of an MEK dominant-negative mutation resulted in a dose-dependent inhibition of sTNFR1 release (Figure 3E), confirming the role of erk1/2 in TACE activation. TNF-α, which failed to induce TNFR1 shedding, did not induce phosphorylation of TACE or erk1/2 (Figure 3F).

Protein A interacts with EGFR

Both TNF-α and protein A signal proinflammatory responses through TNFR1 and the expected TRADD–TRAF2 cascade (Gómez *et al*, 2004). To determine if the protein A–TNFR1–TRAF2 cascade is involved in receptor shedding, we monitored sTNFR1 in cells expressing a TRAF2 dominant-negative mutant (DN) (Figure 4A). Whereas IL-8 production was inhibited (data not shown), shedding was unaffected, indicating that an epithelial receptor distinct from TNFR1 must be involved. In the original analysis of the interactions between protein A and epithelial cells we identified a protein with a molecular weight of approximately 170 kDa, the size of the epidermal growth factor receptor (EGFR), in addition to TNFR1 (Gómez *et al*, 2004). EGFR is apically expressed in the airway epithelial cells, and the signaling cascade that leads to TACE activation resembles EGFR signaling (Zwick *et al*, 1999). Co-immunoprecipitation studies were performed to determine if EGFR directly interacts with protein A (Figure 4B). EGFR was detected by immunoblot in protein A stimulated lysates captured with antibody to protein A but not in control lysates stimulated with BSA. Protein A–EGFR interaction on the apical surface of polarized airway epithelial cells was then demonstrated by confocal imaging (Figure 4C).

A discrete region of protein A, domain D, is sufficient for TNFR1 recognition and IL-8 induction, as well as TNFR1 shedding (Gómez *et al*, 2006). We screened a collection of point mutations in domain D to determine if this region recognizes EGFR. Two mutants (L17A and F5A) that retained the ability to induce IL-8 production (Gómez *et al*, 2006) but failed to induce TNFR1 shedding were identified (Figure 4D). Neither mutant was able interact with EGFR, as demonstrated by co-immunoprecipitation and confocal imaging, indicating that discrete binding domains recognize TNFR1 and EGFR independently (Figure 4B and C). Both mutants stimulated TNFR1 mobilization (Figure 4E) and colocalization with TACE (Figure 4F), indicating that receptor mobilization induced by protein A is independent of EGFR binding, and that the protein A–EGFR interaction is required for TACE activation (Figure 4D).

Protein A–EGFR signaling mediates TACE activation

Activation of EGFR involves both Src-dependent phosphorylation and autophosphorylation of multiple tyrosines (Schlessinger, 2000). EGFR phosphorylation at Tyr1173 was detected as soon as 5 min after protein A stimulation and was blocked by the EGFR tyrosine kinase inhibitors AG1478 and C56 (Figure 5A). Protein A-induced erk1/2 phosphorylation was also blocked by AG1478 and C56, indicating that erk1/2 activation is mediated by EGFR (Figure 5A). The EGFR tyrosine kinase inhibitor C56 significantly blocked TNFR1 shedding (Figure 5B). As expected, the L17A mutant did not induce EGFR phosphorylation (Figure 5C). Erk1/2 was not phosphorylated in response to protein A in the presence of the c-Src inhibitor PP1 (Figure 5D), and both PP1 and PP2, another c-Src inhibitor, decreased sTNFR1 shedding in a dose-dependent manner (Figure 5E). These observations are consistent with the requirement for c-Src in EGFR-mediated TACE activation.

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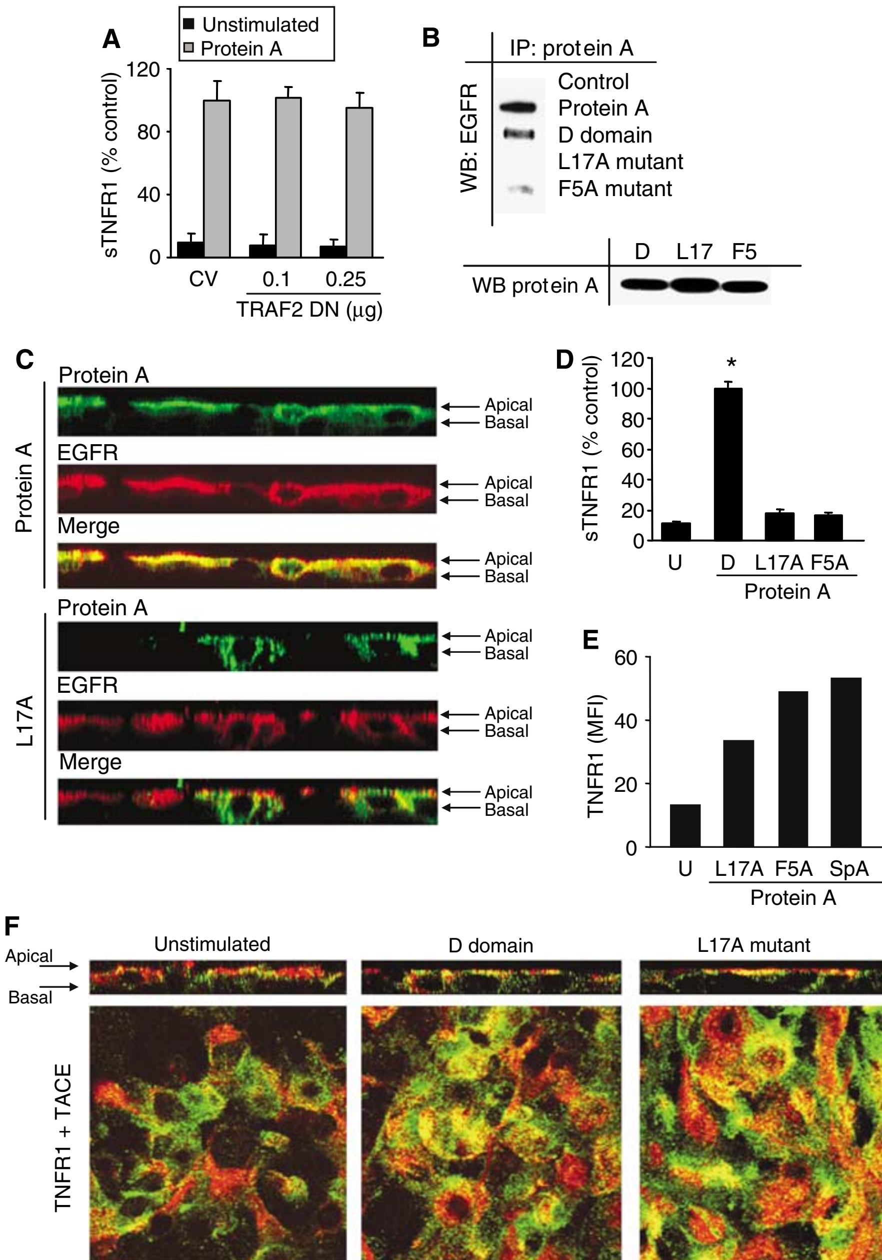

Figure 4 Protein A binding to EGFR. (**A**) 1HAEo− cells were transfected with a TRAF2 dominant-negative (DN) mutant or the control vector (CV) and sTNFR1 was detected by ELISA. Data represent the mean and s.d. of four wells and are expressed as percentage of sTNFR1 measured in stimulated CV transfected cells after protein A stimulation. (**B**) 1HAEo− cells were incubated with bovine serum albumin (control), protein A, protein A domain D or mutated domains (L17A and F5A) for 1 h. Cell lysates were immunoprecipitated (IP) with anti-protein A antibody and immunoblotted (WB) for EGFR. Immunoblots of the D domain and the mutants with the anti-protein A antibody are shown as control. (**C**) Polarized airway epithelial cells were incubated with protein A or the L17A mutant and stained for protein A (green) or EGFR (red). Apical colocalization (yellow) of EGFR and protein A is shown (merge). (**D**) 1HAEo− cells were stimulated with protein A domain D, mutants L17A and F5A or media alone (U: unstimulated) and sTNFR1 was measured by ELISA. Data represent the mean and s.d. of six wells and are expressed as percentage of sTNFR1 induced by protein A domain D (*$P<0.01$, Student's *t* Test). (**E**) 1HAEo− cells were stimulated with the wild-type protein A, the mutants L17A and F5A or media alone (U: unstimulated) for 2 h and cells were stained for surface expression of TNFR1. MFI: mean fluorescence intensity. One representative experiment of three is shown. (**F**) Polarized primary human nasal polyp (HNP) cells grown on transwells, stimulated with protein A domain D, the mutant L17A or media alone (unstimulated) were stained for TACE (green) and TNFR1 (red) and analyzed by confocal imaging. Colocalization of TACE and TNFR1 appears yellow. *Z*-sections and corresponding *x–y* scans of apical sections are shown.

Protein A-induced TNFR1 shedding is not mediated by EGFR ligands

EGFR signaling in the airway cells can be activated through autocrine pathways that involve the induction of TGF-α and other EGFR ligands (Shao and Nadel, 2005; Janes *et al*, 2006; Zhao *et al*, 2006). Protein A binds directly to EGFR and also induces TGF-α production (Figure 6A). However, it is very unlikely that EGFR activation was secondary to TGF-α production because the amount of TGF-α produced by the airway epithelial cells (1 ng/ml) in response to protein A was not sufficient to induce EGFR phosphorylation (data not shown). Moreover, stimulation of the airway epithelial cells with a

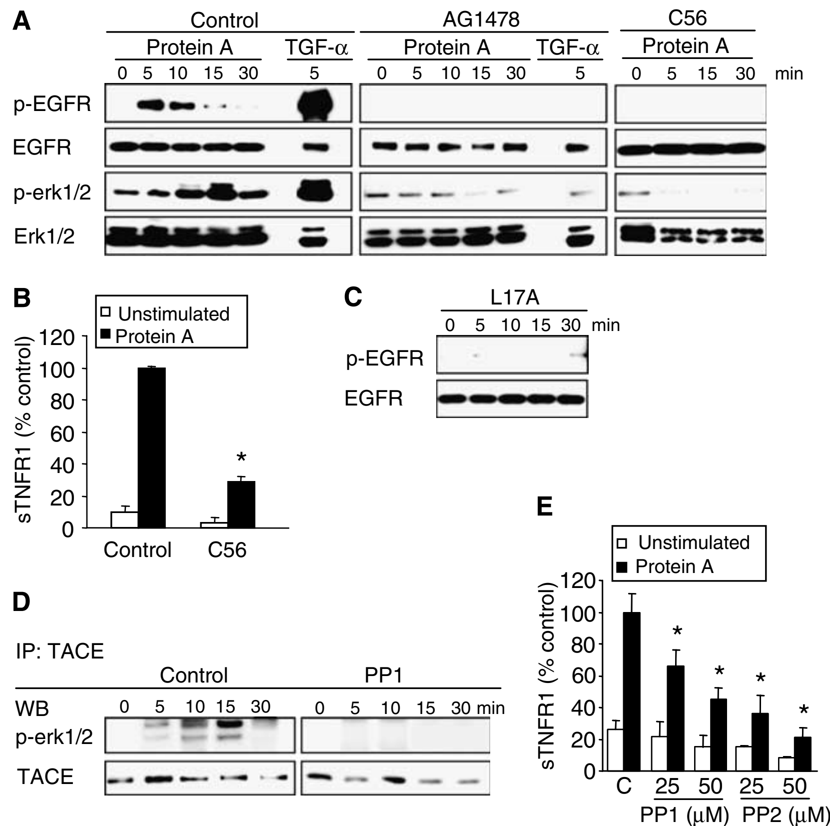

Figure 5 Protein A activation of EGFR signaling. (**A**) Lysates from 1HAEo− cells collected after exposure to protein A or TGF-α in the presence or absence of the EGFR tyrosine kinase inhibitors AG1478 or C56 were immunoblotted for phospho-EGFR (p-EGFR) and phospho-erk1/2 (p-erk1/2). (**B**) 1HAEo− cells were stimulated with protein A in the absence (C: control) or presence of the EGFR tyrosine kinase inhibitor C56 and sTNFR1 was measured by ELISA. Data represent the mean and s.d. of six wells and are expressed as percentage of sTNFR1 detected after stimulation with protein A in the absence of inhibitor (*$P<0.01$, Student's t Test). (**C**) Lysates from 1HAEo− cells collected after exposure to the protein A mutant L17A were screened for phospho-EGFR (p-EGFR). (**D**) TACE immunoprecipitates (IP) from cell lysates stimulated with protein A in the presence or absence of the c-Src inhibitor PP1 were immunoblotted (WB) for phospho-erk1/2 (p-erk1/2) and erk1/2. (**E**) 1HAEo− cells were stimulated with protein A in the absence (C: control) or presence of the c-Src inhibitors PP1 and PP2 and sTNFR1 was measured by ELISA. Data represent the mean and s.d. of six wells and are expressed as percentage of sTNFR1 detected after stimulation with protein A in the absence of inhibitors (*$P<0.001$, Student's t Test).

10-fold increased concentration of TGF-α (10 ng/ml) did not induce TNFR1 shedding (Figure 6B). We also excluded autocrine stimulation by other EGFR ligands by demonstrating that protein A induced EGFR phosphorylation in the presence of TAPI-1, a metalloprotease inhibitor that prevents the release of EGFR ligands by TACE (Figure 6C).

Discussion

Staphylococci are common human pathogens, that target a broad range of eukaryotic tissues. While *S. aureus* interactions with many host matrix components facilitate the attachment stage of pathogenesis, their ability to exploit the immune system enables them to persist and thrive, even in the presence of a normal immune response. Protein A expression appears to play an important role in the success of *S. aureus* as a human pathogen. Most bacterial pathogens elicit proinflammatory signaling that stimulates the influx of neutrophils to eradicate infection. The host, then, must regulate chemokine and cytokine responses appropriately. However, in addition to its potent immunostimulatory activity, *S. aureus*, through the expression of protein A, has also evolved complex mechanisms to regulate TNFR1 signaling. Protein A effectively activates TACE, which is targeted to

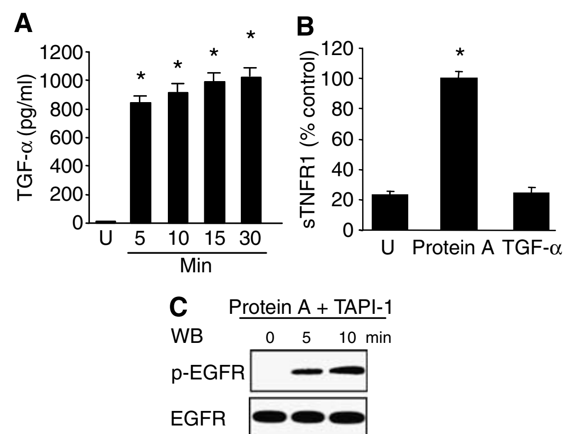

Figure 6 Role of EGFR ligands in TNFR1 shedding. (**A**) 1HAEo− cells were incubated with protein A or media alone (U: unstimulated) and TGF-α was detected in culture supernatants by ELISA. Data represent the mean and s.d. of sextuplicated wells (*$P<0.01$, Student's t Test). (**B**) 1HAEo− cells were stimulated with protein A, TGF-α or media alone (U: unstimulated) and sTNFR1 was measured by ELISA. Data represent the mean and s.d. of six wells and are expressed as percentage of sTNFR1 induced by protein A (*$P<0.01$, Student's t Test). (**C**) Lysates from 1HAEo− cells collected after exposure to protein A in the presence of the TACE inhibitor TAPI-1 were immunoblotted for phospho-EGFR (p-EGFR) and EGFR as control.

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newly mobilized TNFR1, inducing its release from the cell surface.

The binding of protein A to the Fc region of IgG, the Fab region of immunoglobulin of the VH3 subclass (Moks *et al*, 1986; Sasso *et al*, 1989; Roben *et al*, 1995; Jansson *et al*, 1998), the von Willebrand factor (Hartleib *et al*, 2000) and TNFR1 (Gómez *et al*, 2006) have been mapped. The protein A–TNFR1 interaction mimics the pathway stimulated by the natural ligand TNF-α. However, the same IgG-binding domain of protein A also has novel interactions with epithelial cells that are not analogous to those of the endogenous ligand TNF-α. Although TNF-α–TNFR1 activation and protein A–TNFR1 ligation both stimulate an identical proinflammatory signaling cascade (Gómez *et al*, 2004), protein A also interacts with EGFR to induce phosphorylation and activation of TACE.

The involvement of EGFR, ubiquitously expressed on the airway cells, in mediating TNFR1 shedding via TACE was unanticipated. Although there are several reports detailing TACE activation to cleave ligands involved in subsequent autocrine stimulation of EGFR (Shao and Nadel, 2005; Janes *et al*, 2006; Zhao *et al*, 2006), we show instead, that a protein A–EGFR interaction stimulates phosphorylation of both EGFR and TACE itself. Although the specific EGFR locus that is recognized by protein A was not mapped, we show by co-immunoprecipation and confocal imaging that protein A and EGFR are closely associated. Moreover, we identified protein A mutants capable of activating IL-8 signaling that are unable to activate EGFR, indicating a discrete interaction, separate from the proinflammatory signal. This protein A–EGFR interaction is required for receptor shedding, as mutants unable to bind EGFR fail to induce EGFR activation and TNFR1 cleavage. EGFR is also a target of an autocrine system stimulated by the epithelial production of TGF-α. Although protein A induces TGF-α production, this cytokine by itself failed to induce receptor shedding, and the interaction of protein A with EGFR is sufficient to activate TACE in the presence of a metalloprotease inhibitor that prevents TGF-α release.

Several pathways that initiate TACE activity have been identified in the airway epithelial cells (Shao *et al*, 2004; Shao and Nadel, 2005; Kuwahara *et al*, 2006). The phosphorylation of TACE by erk1/2, induced by protein A, is also induced by phorbol esters (Doedens *et al*, 2003) or ROS (Hino *et al*, 1999; Zhang *et al*, 2001; Shao *et al*, 2004) in other cell systems. The signaling pathways initiated by the activation of TNFR1, however, are insufficient to activate TACE and induce TNFR1 shedding, as shown in cells where TNFR1 signaling was blocked through the expression of a TRAF2 DN mutant. Moreover, TNF-α alone is an insufficient stimulus to induce TACE activation. Alternative routes of TACE activation, such as TGF-α–EGFR signaling were also insufficient to induce TNFR1 shedding. It appears that mobilization of TNFR1, induced by protein A but not by TNF-α, is necessary to deliver the receptor to the cell surface, where it becomes a substrate for the activated TACE. As TACE has many potential substrates, the mobilization of TNFR1 in conjunction with TACE phosphorylation may serve to target activated TACE to this specific substrate. The domains of protein A that are involved in receptor mobilization are yet to be identified.

The interaction between protein A, a single bacterial virulence factor, and EGFR, provides a novel mechanism to regulate TNFR1 availability. Other human pathogens also exploit EGFR signaling to evade immune responses. Members of the Herpes virus family have several interactions with EGFR that contribute to pathogenesis. Binding of the cytomegalovirus glycoprotein B to EGFR induces receptor phosphorylation and contributes to viral invasion (Wang *et al*, 2003b; Compton, 2004). Similarly, the Epstein-Barr virus latent membrane protein 1 (LMP1) activates EGFR phosphorylation to induce cell proliferation (Miller *et al*, 1997; Brinkmann and Schulz, 2006). Of note, protein A shares conserved sequence domains with LMP1, indicating that Staphylococci may be using a similar strategy to exploit host signaling cascades without inducing responses that would be deleterious to either the host cell or the organism.

These studies provide further evidence to demonstrate the contribution of protein A to the success of *S. aureus* as a human pathogen. It is an exceptional virulence factor, a single protein that can target multiple immunologically important eukaryotic receptors. It is probably not a coincidence that protein A is among the most highly conserved staphylococcal virulence factors expressed, nor that its levels of expression are significantly increased in staphylococci isolated from invasive human infections.

Materials and methods

Cell lines, bacterial strains and reagents

1HAEo− and 16HBE cells (human airway epithelial lines) (D Gruenert, Pacific Medical Center Research Institute, San Francisco, California) were grown as previously described (Rajan *et al*, 2000; Ratner *et al*, 2001) Primary airway epithelial cells isolated from human nasal polyps (HNP) were grown on Transwell-clear filters (Corning Costar) in M3 medium as previously described (DiMango *et al*, 1998). RAW cells (a murine macrophage line) were grown in RPMI medium supplemented with 10% fetal calf serum (Invitrogen). Protein A from *S. aureus* Newman and the IgG binding domain D were cloned and purified as a GST-fusion protein, resuspended in PBS and used at a concentration of 2.5 μM for stimulation. Mutations were introduced into SpA domain D using a PCR-based mutagenesis strategy. The amino-acid substitutions F5A and L17A were selected for this study among 10 constructed (Gómez *et al*, 2006). TNF-α and TGF-α (Calbiochem) were used for stimulation at 100 ng/ml and 10 ng/ml, respectively. Brefeldin A (Sigma-Fluka) was used at 10 μg/ml and cycloheximide (Sigma) was used at 10 μg/ml. TAPI-1 (Calbiochem) was used at 50 μM.

Flow cytometry

Cells were stimulated with protein A or TNF-α, washed three times and stained with anti-TNFR1 (H-271) or anti-TACE (C-15) polyclonal antibodies (Santa Cruz Biotech). Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) was used. Cells were then washed, fixed in 1% paraformaldehyde and analyzed with a Becton Dickinson FACS Calibur. Data were collected using Cell Quest software and analyzed with Winmdi.

IL-8, TGF-α and sTNFR1 ELISA

Cells were weaned from serum for 24 h and exposed to protein A, TNF-α or TGF-α for 4 h unless indicated. IL-8 (BD pharmingen), TGF-α and sTNFR1 (R&D Systems) in the supernatant were measured by ELISA. The effect of MEK, c-Src and EGFR tyrosine kinase inhibitors was tested by pretreating the cells for 30 min with 10 μM UO126 (MEK, Calbiochem), 50 μM PP1 (c-Src, Biomol), 50 μM PP2 (c-Src, Calbiochem), 10 μM AG1478 (EGFR tyrosine kinase, Calbiochem) or 50 μM compound 56 (EGFR tyrosine kinase, Calbiochem) and adding fresh inhibitors during stimulation. The effect of MEK and TRAF2 DN mutants was tested by transfecting 1HAEo− cells grown to 50–70% confluence with HMEK (K97R) (Adamo *et al*, 2004) TRAF2 DN (Gómez *et al*, 2004) or a vector control using FuGene6.0 (Roche). After 16 h, cells were weaned from serum for 24 h and stimulated with protein A.

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Immunoprecipitation and Western Blot

Cells were lysed using 60 mM *n*-octyl-β-D-glucopyranoside in TBS (0.1 M Tris–HCl and 0.15 M NaCl (pH 7.8)) containing Complete Mini protease inhibitor tablets (Roche), 1 mM sodium orthovanadate, 100 mM sodium fluoride and 20 μM GM6001. For protein A–EGFR co-immunoprecipitation, lysates (500 μg of protein) from cells stimulated with bovine serum albumin (control), protein A, protein A domain D or the L17A and F5A mutants were incubated with the monoclonal antibody to protein A (Sigma) overnight at 4°C with shaking. For TACE immunoprecipitations, cell lysates (300 μg of protein) were incubated with 1 μg of goat anti-TACE (C-15) antibody (Santa Cruz Biotech) overnight at 4°C with shaking. Protein G agarose beads were then added for 1 h at 4°C with shaking. Beads were washed twice with 500 mM NaCl, 50 mM Tris and 1% NP-40, followed by a wash with 20 mM Tris and resuspended in NUPAGE sample buffer and reducing agent (Invitrogen). Proteins were separated on 4–12% bis–tris NUPAGE gels (Invitrogen), transferred to PVDF Immobilon P membrane (Millipore) and blocked with 5% milk in TBST (50 mM Tris pH 7.5, 150 mM NaCl and 0.05% Tween) for 1 h at room temperature. Immunodetection was performed using anti-phospho-Threonine (Cell signaling), anti-phospho-erk1/2, anti-erk1/2, anti-phospho EGFR (Tyr 1173), anti-EGFR (1005) or anti-TACE (C-15) (Santa Cruz Biotech) antibodies, followed by secondary antibodies conjugated to horseradish peroxidase (Santa Cruz). Anti-TACE antibody C-15 recognized both the pro-form and the mature form (devoid of the pro-domain) of TACE in the airway epithelial cells. The identity of these bands in the airway cell lines used was confirmed by using the anti-TACE antibody H-300 that only recognizes the pro-form of TACE.

RNA interference

Inhibition of TACE expression in the 16HBE airway epithelial cells was previously described (Gómez *et al*, 2005). Briefly, two cell lines were constructed by using two pairs of oligonucleotides containing 19 bp of human TACE were generated as follows: pair 1: 5′gatccccGTAAGGCCCAGGAGTGTTTttcaagagaAAACACTCCTGGGCCTTACttttggaaa3′ and 5′agcttttccaaaaGTAAGGCCCAGGAGTGTTTtctcttgaaAAACACTCCTGGGCCTTACggg3′; pair 2: 5′gatccccCATAGAGCCACTTTGGAGAttcaagagaTCTCCAAAGTGGCTCTATGttttggaaa3′ and 5′agcttttccaaaaCATAGAGCCACTTTGGAGAtctcttgaaTCTCCAAAGTGGCTCTATGggg3′. To construct pRS-TACE-1 and pRS-TACE-2, the oligos were annealed and ligated into *Bgl*II and *Hin*dIII sites of the pRetroSuper vector (pRS) (Brummelkamp *et al*, 2002). Construct integrity was confirmed by direct sequencing of the plasmid. Packaging of retroviral constructs was carried out in HEK293T cells (Pear *et al*, 1993). 16HBE cells were infected for 18 h in the presence of 4 mg/ml polybrene (Sigma). pBabe-puro-EGFP was used to monitor the efficiency of transfection to 293T cells and infection. A pRS-scramble plasmid (pRS-sc) was used as a control by cloning the sequence ggcagttccaccccagtgc into pRS as described for pRS–TACE.

Confocal microscopy

1HAEo−, 16HBE or primary airway epithelial cells isolated from human nasal polyps (HNP) were grown on Transwell-Clear filters (Corning-Costar) with an air–liquid interface to form polarized monolayers. Protein A stimulated and control unstimulated cells were fixed with 4% paraformaldehyde and after blocking with 5% normal serum, rabbit polyclonal anti-TNFR1 or goat polyclonal anti-TACE antibodies (Santa Cruz Biotech) were added for 1 h. For protein A binding experiments, cells were fixed as above and incubated with the full-length protein A, protein A domain D or the mutant L17A for 1 h at room temperature. After washing, rabbit polyclonal anti-EGFR and monoclonal antibody to protein A were added for 1 h. For Golgi colocalization experiments, cells were incubated prior to stimulation with 5 uM of the Golgi probe BODIPY TR (Molecular Probes) for 30 min at 4°C. Cells were preincubated with the media or brefeldin for 30 min and stimulated in the presence or absence of brefeldin for 2 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-100 and then blocked and stained for TNFR1 as described above. Alexa Fluor 488- and 594-conjugated secondary antibodies (Molecular Probes) were used. After washing, filters were removed from transwells and mounted with Vectashield (Vector Laboratories Inc.) onto glass slides.

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