The *Escherichia coli* AIDA autotransporter adhesin recognizes an integral membrane glycoprotein as receptor

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The AIDA-I autotransporter adhesin, as a prototype of the AIDA adhesin family, represents a tripartite antigen consisting of the functional adhesin AIDA-I (α-domain), which mediates the specific attachment of bacteria to target cells, and a two-domain translocator (AIDA") organized in the β₁- and β₂-domains. Cellular receptor moieties for the adhesin AIDA-I have not been identified. Here, it is demonstrated that the purified adhesin binds specifically to a high-affinity class of receptors on HeLa cells. Additionally, the adhesin was found to bind to a variety of mammalian cell types, indicating a broad tissue distribution of the receptor moiety. By using complementary techniques, including co-immunoprecipitation and one- and two-dimensional gel electrophoresis, the AIDA-I binding protein on HeLa cells was identified as a surface glycoprotein of about 119 kDa (gp119). The gp119 AIDA-I cellular receptor protein was characterized biochemically and found to be an integral N-glycosylated membrane protein with a pl of 5-2.

**INTRODUCTION**

*Escherichia coli* strains remain a major cause of acute and persistent diarrhoea; they not only contribute to the high mortality rate among infants in developing countries but they also represent a growing health problem in industrialized regions (Nataro & Kaper, 1998). Diffusely adhering *E. coli* (DAEC) strains have been particularly associated with persistent diarrhoea in developing countries, affecting especially children older than one year of age (Baqui et al., 1992; Giron et al., 1991; Levine et al., 1993), and represent one of the six classes of diarrhoeagenic *E. coli* (Kaper, 1998; Nataro & Kaper, 1998). However, as the term DAEC encompasses many different phenotypes, the DAEC strains associated with outbreaks of diarrhoea have to be investigated in more detail in order to be able to define an additional trait that is more specific than diffuse adherence.

Thus far, four distinct adhesins have been identified in DAEC strains that mediate the diffuse-adhering phenotype, allowing organisms to adhere to cultured epithelial cells. The DAEC strain C1845 (Bilge et al., 1989) expresses a fimbrial adhesin, F1845, which belongs to the Dr family (Nowicki et al., 1990) and recognizes the decay-accelerating factor CD55 as cellular receptor (Bernet-Camard et al., 1996). Two other adhesins, namely a 57 kDa protein (Yamamoto et al., 1996) and CF16K (Jallat et al., 1994), have also been reported.

**Abbreviations:** AIDA, adhesin involved in diffuse adherence; CD, circular dichroism; GPI, glycosylphosphatidylinositol; HRP, horseradish peroxidase; PI-PLC, phosphatidylinositol-specific phospholipase C.

AIDA (adhesin involved in diffuse adherence) has been isolated and characterized from the *E. coli* diarrhoea isolate 2787 (O126 : H27) (Benz & Schmidt, 1989, 1992a, b; 2001; Konieczny et al., 2000, 2001; Niewerth et al., 2001; Suhr et al., 1996). As demonstrated by our and other laboratories, AIDA belongs to the growing family of autotransporter proteins (Henderson et al., 1998, 2000; Henderson & Nataro, 2001; Henderson & Owen, 1999; Jose et al., 1995; Suhr et al., 1996). Two genes (aah and aidA) are necessary for full adherence activity. They have been localized to the larger of the two ~100 kb plasmids harbouring by the clinical isolate. *aidA* encodes a pre-pro-protein of 132 kDa, which, after N- and C-terminal processing, matures to the adhesin AIDA-I (~100 kDa, α-domain) and the (auto-catalytically) cleaved membrane-integrated β-domain of 47-5 kDa (Benz & Schmidt, 1992a, b; Suhr et al., 1996). AIDA-I remains non-covalently associated with the bacterial surface. To be fully functional, the adhesin needs to be post-translationally modified by heptose residues at multiple sites. This modification is mediated by the activity of the *aah* gene product, the ‘autotransporter adhesin heptosyltransferase’ (Benz & Schmidt, 2001). The β-domain directs the outer-membrane localization and translocation to the bacterial surface of the authentic N-terminal adhesin (α- or ‘passenger’ domain) or of heterologous passenger (poly-) peptides (Konieczny et al., 2000; Maurer et al., 1997; Suhr et al., 1996). Thus, the β-domain has been termed the ‘translocator’ (Konieczny et al., 2000, 2001).

Bacterial adhesion mediated by fimbrial or afimbrial adhesion systems is regarded as the first step in infection...
(Finlay & Falkow, 1997; Klemm & Schembri, 2000; Westerlund & Korhonen, 1993), contributing to tissue tropism (Hultgren et al., 1993; Lindstedt et al., 1991). Although numerous bacterial adhesins have been characterized, for the majority of adhesins the identity of the corresponding host receptors has remained obscure. Most bacterial adhesin receptors that have been elucidated represent carbohydrate structures of glycolipids or glycoproteins (Giannasca et al., 1996; Hultgren et al., 1993; Karlsson, 1989; Mouricout, 1997; Salam Khan et al., 2000; Sharon, 1987). Recently, however, some cellular membrane proteins have been described as potent receptors mediating adherence and subsequent internalization, e.g. $\beta_1$-integrins for the invasin of *Vesirina pseudotuberculosis* (Ibsberg & Leong, 1990), $\alpha_2\beta_1$-integrin for Ipa proteins of *Shigella flexnerii* (Watarai et al., 1996), E-cadherin for the intercellular of *Listeria monocytogenes* (Mengaud et al., 1996) and CD66 for Opa proteins of *Neisseria* spp. (Chen & Gotschlich, 1996; Chen et al., 1997; Virji et al., 1996).

In this study, we investigated the binding of the AIDA autotransporter adhesin AIDA-I to mammalian cells and identified the AIDA-I receptor. Investigation of the binding properties of AIDA-I with different mammalian cell lines derived from various tissues and species demonstrated the specific binding and an ubiquitous distribution of the candidate receptor molecule. By co-immunoprecipitation and biochemical characterization we identified a 119 kDa integral membrane glycoprotein as the AIDA-I receptor; this receptor appears to be distinct from previously described cellular receptors for bacterial adhesins.

**METHODS**

**Bacterial strains, cell lines and culture conditions.** The AIDA autotransporter was isolated from the recombinant laboratory *E. coli* K-12 strain C600/pIB264 expressing the functional adhesin AIDA-I as described previously (Benz & Schmidt, 1992a). Bacteria were grown overnight in Standard I medium (Merck; 150 r.p.m., 37 °C) containing 100 μg ampicillin ml$^{-1}$ for the recombinant strain. Strains were routinely stored at −70 °C in Standard I medium with 15% (v/v) glycerol. HeLa (ATCC CCL-2; human cervical epitheloid carcinoma), INT-407 (ATCC CCL-6; human embryonic intestine) and CV-1 (ATCC CCL-70; fibroblast-like cells, African green monkey kidney) cells were routinely grown at 37 °C in a 10% CO$_2$ atmosphere in Dulbecco's modified Eagle's medium (DMEM; PAA) supplemented with 10% (v/v) fetal calf serum (FCS), containing 1 mM glutamine, 100 U penicillin ml$^{-1}$ and 100 μg streptomycin ml$^{-1}$. Caco-2 cells (DSM ACC 169; human colon adenocarcinoma) were grown at 37 °C in a 5% CO$_2$ atmosphere in Eagle's minimal essential medium (EMEM; PAA) supplemented with 10% (v/v) FCS, containing 25 mM glucose, 2 mM glutamine, 1% (v/v) non-essential amino acids, 100 U penicillin ml$^{-1}$ and 100 μg streptomycin ml$^{-1}$. CHO-K1 cells (ATCC CCL-61; Chinese hamster ovary cells) were grown at 37 °C in a 10% CO$_2$ atmosphere in α-MEM (Biochrom) supplemented with 10% (v/v) FCS, 1 mM glutamine, 100 U penicillin ml$^{-1}$ and 100 μg streptomycin ml$^{-1}$.

**SDS-PAGE, two-dimensional electrophoresis and Western blot analysis.** Prior to separation of proteins by SDS-PAGE, samples were centrifuged (14000 g, 1 min). Proteins were either stained with Coomassie brilliant blue or electroblotted onto nitrocellulose membranes (Schleicher and Schuell). After blocking with 3% (w/v) BSA in D-PBS (Dulbecco's PBS: 8 mM sodium phosphate, 2 mM potassium phosphate, 0-14 M sodium chloride, 0-01 M potassium chloride, pH 7-4) containing 0-1% (v/v) Tween 20, biotinylated proteins were detected with horseradish peroxidase (HRP)-conjugated streptavidin (streptavidin–HRP; Roche Diagnostics) diluted 1:5000 in Tris-buffered saline (TBS)/0-3% BSA/0-1% Tween 20 by chemiluminescence (Pierce). For two-dimensional electrophoresis, the first-dimension isoelectric focusing was performed using 11 cm pre-cast immobilized pH gradients (pH 3–10; Pharmacia) for 16 h at 15 °C according to the modifications introduced by Rabilloud et al. (1997). For the second dimension, SDS-PAGE was performed on 7-5% gels. Proteins were visualized either with silver staining or for biotinylated proteins after Western blotting with streptavidin–HRP.

**Isolation and purification of AIDA-I.** The adhesin AIDA-I was isolated and purified essentially as described previously (Benz & Schmidt, 1992a). Briefly, bacteria from overnight cultures were pelleted and resuspended in 1/10 volume with PBS; the adhesin was selectively detached from the bacterial surface by mild heat extraction (20 min, 60 °C). The bacteria-free supernatant was concentrated to a volume of 1/5–1/10 by reverse dialysis (4-6 kDa pore size) with polyethylene glycol (PEG; Sigma) 15000–20000 Da followed by ultracentrifugation (160 000 g, 2 h, 4 °C) to remove micelles derived from bacterial membranes. The supernatant was loaded onto a Sephacryl S300 HR gel filtration column (10–1500 kDa separation range, diameter 1-6 cm, 183 ml gel bed volume; Pharmacia) and run with 50 mM sodium phosphate buffer (pH 7-4) containing 100 mM NaCl with a flow rate of 0-4–0-6 ml min$^{-1}$. Fractions of 1-5–2 ml were collected and protein was detected by measuring the absorbance at 280 nm. Fractions containing pure AIDA-I as determined by SDS-PAGE were pooled and concentrated by a second reverse PEG dialysis. Protein concentration was determined by the method of Bradford (1976) as modified by Read & Northcote (1981). Aliquots were snap-frozen in liquid nitrogen and stored at −70 °C.

**Circular dichroism (CD) spectroscopy.** The CD spectrum of a 0-1 μM solution of AIDA-I was recorded at 20 °C using a Jobin-Yvon CD6 spectral polarimeter in 50 mM sodium phosphate, pH 7-4, with a scan speed of 20 nm min$^{-1}$ at a resolution of 0-5 nm and a sensitivity of 188 mdeg under protective nitrogen gas (light pathway 0-1 mm). The spectrum was accumulated fivefold, corrected for buffer contributions and converted to mean residue ellipticities according to Schmid (1989). Data were analysed for secondary structure content using the manufacturer’s CDNN software.

**Generation of specific polyclonal AIDA-I antibodies.** Specific antibodies were raised against purified AIDA-I protein in a female New Zealand White rabbit. After collecting pre-immune serum, a total of five immunizations (2–4 weeks apart) with purified AIDA-I using the MPL + TDM + CWS adjuvants system (Sigma) was performed. Sera were collected 10 days after the fourth and fifth immunization according to the method of Harlow & Lane (1988).

**Detection of AIDA-I binding by immunofluorescence.** HeLa, Caco-2, INT-407, CV-1 and CHO-K1 cells were seeded at a density of $8 \times 10^4$ per well in 24-well tissue culture plates on glass coverslips and grown overnight to 70–90% confluency. Cells were washed twice with D-PBS containing 0-9 mM MgCl$_2$ and 0-5 mM CaCl$_2$ (D-PBS/Mg$^{++}$/Ca$^{++}$) before being fixed for 20 min with 4% (w/v) paraformaldehyde in D-PBS/Mg$^{++}$. Following an additional wash with D-PBS, potentially reactive sites were quenched with 0-2% glycine in D-PBS. Non-specific binding sites were blocked with 1-5% BSA in D-PBS for 30 min.
For the detection of AIDA-I receptors, β1-integrins (CD29), and/or decay-accelerating factor (CD55), fixed cells were sequentially incubated with (i) 5 μg ml⁻¹ purified adhesin in 0-1 % BSA/D-PBS for 1 h, (ii) rabbit polyclonal anti-AIDA-I serum (diluted 1:10 000) in 0-1 % BSA/D-PBS combined with 10 μg ml⁻¹ mAbs specific for CD29 (clone 9E10, UBI) or CD55 (clone BRIC110, Serotec) for 1 h, and (iii) a mixture of Texas-red-conjugated goat anti-rabbit and fluorescein (DTAF)-conjugated goat anti-mouse immunoglobulin G antibodies (diluted 1:100 in 0-1 % BSA/D-PBS; Dianova) for 1 h. Three washes in D-PBS containing 0-01 % Triton X-100 followed each incubation step. For immunodetection on viable cells, AIDA-I (50 μl of 5 μg ml⁻¹ stock solution in 0-1 % BSA/DMEM) was incubated for 1 h at 37 °C and 10 % CO₂ in DMEM prior to fixation.

Stained specimens were mounted in Moviol/DABCO and analysed with a Zeiss LM410 confocal laser scanning microscope equipped with an argon–krypton gas laser (pinhole 20 nm, data files represent the mean of sequential eight times recording of all three channels) or a Leica DM-RXA fluorescence microscope connected to a cooled one-chip CCD camera (Princeton Instruments).

Cell-surface ELISA and modification of cell-surface antigens. HeLa cells were seeded in 96-well Primaria tissue culture plates (Falcon; Becton Dickinson) at a density of 3×10⁴ cells in 200 μl per well and grown overnight to about 90–100 % confluency. Preparation of cell monolayers and immunodetection of antigens were performed essentially as described above with the following modifications. Cells were fixed with 2-5 % (v/v) paraformaldehyde/0-2 % (v/v) glutaraldehyde/D-PBS/Mg²⁺ for 20 min, quenched and blocked with 10 % BSA/D-PBS for 2 h. All incubation steps were carried out at room temperature for 2 h to reach binding equilibrium followed by four to six washings. AIDA-I (concentrations as indicated) was detected with specific rabbit antiserum (1:10 000) followed by peroxidase-conjugated goat anti-rabbit immunoglobulin G (diluted 1:4000; Dianova). Cholera toxin (5 μg ml⁻¹; List Biological Laboratories) was detected with specific mouse antiserum (1:4000; kindly provided by W. Walz-Schmidt, ZMBE). A mAb (1 μg ml⁻¹) was used to detect CD29 (DE6; Biomol) followed by HRP-conjugated secondary anti-mouse antibodies (Dianova). Biotinylated lectins (5 μg ml⁻¹) were analysed with HRP-conjugated streptavidin (1:160 000) and quantified using a colorimetric ELISA assay (Roche Diagnostics) at 405 nm in a microplate reader (Molecular Devices). All experiments were performed in triplicate or quadruplicate. Non-linear regression analysis of kinetic and dose-dependent binding was performed using GraphPad Prism software.

Surface carbohydrates were partially destroyed by incubation of fixed monolayers with 10 mM sodium metaperiodate (Sigma) in 50 mM sodium acetate (pH 4-5) for 1 h at room temperature in the dark (Falk et al., 1994; St Gene, 1994; Woodward et al., 1985). After rinsing the monolayers twice with D-PBS, they were reduced with 50 mM sodium borohydride in D-PBS (pH 7-4) for 30 min at room temperature followed by rinsing in D-PBS. Controls were treated identically except that periodate was omitted from the sodium acetate buffer.

For inhibition studies, a panel of biotinylated lectins was employed: Solanum tuberosum [STA; GlcNAc-(1,4)-GlcNAc], Canavalia ensiformis [ConA; Man, Glc, GlcNAc, branched N-glycosides], Arachis hypogaea [PNA; Gal-(1,3)-GalNAc], Erythrina cystagalli [ECA; Gal-(1,4)-GalNAc], Helix pomata [HPA; Gal, GalNAc], Vicia villosa [VVA; GalNAc-(1,3)-Gal], Triticum vulgaris [WGA; GlcNAc-(1,4)-GlcNAc], Maackia amurensis [MAA; NANA-(2,3)-Gal], Sambucus nigra [SNA; NANA-(2,6)-Gal/GalNAc] and Ulex europaeus [UEA 1; Fuc-(1,2)-Gal-(1,4)-GlcNAc]. Lectins were obtained either from Sigma or Vector Laboratories. Inhibition studies were performed according to the procedure described for the cell-surface ELISA. Biotinylated lectins were used at 20 μg ml⁻¹ (10-fold excess over AIDA-I).

For proteolytic digestion of surface-exposed proteins, monolayers were treated with 100 μg proteinase K ml⁻¹ (Roche Diagnostics) in D-PBS at room temperature for 1 h. To inhibit residual proteinase activity, monolayers were thoroughly rinsed with D-PBS containing 5 mM PMSF, 1 mM Pefabloc and 1 % FCS.

Cell-surface biotinylation. This was essentially performed as described by Gottardi & Caplan (1992) and Kähne & Ansorge (1994) with some modifications. Briefly, HeLa cells were seeded into 10 cm Petri dishes and grown overnight to 80–100 % confluency (about 3·5×10⁶ cells). All following steps were performed at 4 °C using pre-cooled reagents and solutions. Cells were washed three times with D-PBS/Mg²⁺ and surface-labelled for 30 min with freshly prepared 250 μg NHS-S-biotin ml⁻¹ (Pierce) from a stock solution (100 mg ml⁻¹ in DMSO) in 10 mM triethanolamine containing 0-8 % (w/v) NaCl and 0-02 % (w/v) KCl (pH 9-0). Residual unreacted biotin was removed by washing twice with DMEM (5 min each) followed by two additional washing steps with TBS (pH 7-4).

Cell extraction, cell fractionation and immunoprecipitation. Cells were lysed directly in 1 ml solubilization buffer [50 mM sodium phosphate, pH 7-4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 % (v/v) glycerol, 0-025 % Na₂SO₄, 10 μg leupeptin ml⁻¹, 1 μg aprotinin ml⁻¹, 1 mM Pefabloc, 0-125 U 2 μg-macroglucosaminidase ml⁻¹] containing 2 % (w/v) CHAPS. Lysates were incubated using an end-over-end rotor. After 30 min solubilization, 1·2 ml lysates were pre-cleared with 30 μl Protein A-Sepharose for 30 min and split into two equal fractions. Each fraction was adjusted to 1·2 ml with Tris saline acid solution (TSA: 50 mM Tris/HCl, pH 7-4, 100 mM NaCl, 0·02 % Na₂SO₄) to dilute the detergent. Following 2 h incubation either with or without 5 μg AIDA-I or anti-CD29/55 mAbs, 30 μl Protein A-Sepharose (Pharmacia) loaded with 25 μl AIDA-I-specific antiserum or Protein G-Sepharose (Pharmacia) was added and incubated for an additional 2 h. The immune complexes were recovered by a brief centrifugation (14 000 g, 1 min) and washed sequentially with TSA, 0-1 % CHAPS/TSA, 0-1 % CHAPS/TSA with 250 mM NaCl, and finally again with TSA. SDS-PAGE loading buffer (30 μl; 10 % glycerol, 1·5 % SDS, 4 % 2-mercaptoethanol, 30 mM Tris/HCl, pH 6-8) was added and the suspension was heated at 100 °C for 5 min.

For cell fractionation and the separation of integral membrane proteins from peripheral membrane-associated proteins, cells were scraped from the culture plates in 1 ml D-PBS containing 1 mM Pefabloc and 10 μg leupeptin ml⁻¹ and sonicated for 4 times for 2 s to rupture the cell membranes. The sonicate was ultracentrifuged (108 000 g, 30 min, 4 °C) and the resulting supernatant containing the soluble cytosolic proteins and the pellet containing membranes, nuclei as well as cytoskeletal components was washed once with D-PBS. Subsequently, the pellet was resuspended in 500 μl of 0·05 M Tris/HCl (pH 7-4), 0·1 M Na₂CO₃ (pH 11), 1 M NaCl, 2 M NaCl or 0·1 M Na₂CO₃/1 M NaCl (pH 11) and incubated for 30 min on ice. Released membrane-associated proteins in the supernatant were separated from integral membrane proteins by ultracentrifugation. Proteins remaining in the pellet were solubilized with 2 % (w/v) CHAPS in solubilization buffer and again subjected to ultracentrifugation. Extracts were diluted either with D-PBS or double-distilled H₂O to adjust to neutral pH and a salt concentration of 150–400 mM before co-immunoprecipitation.

Treatment of co-immunoprecipitates with glycosidases. To investigate a potential glycosylation of the AIDA-I receptor (gp119), lysates from 8-75×10⁶ HeLa cells were divided into five equal-sized
aliquots and gp119 was co-immunoprecipitated. The precipitate was treated with the appropriate enzyme while still bound to the Protein A–Sepharose beads loaded with AIDA-I and specific antiserum. Beads were boiled for 5 min in 10 μl of 2 % SDS. Twenty microlitres of 10 % Nonidet P-40 was added and the volume was adjusted to 200 μl with 50 mM sodium phosphate buffer (pH 7.2) to dilute the SDS concentration. Separate aliquots were incubated at 37 °C overnight with the addition of the following enzymes: (i) 20 U peptide-N-glycosidase F ml⁻¹ (Roche Diagnostics), (ii) 25 mU O-glycosidase ml⁻¹ (Roche Diagnostics), (iii) 20 mU sialidase ml⁻¹ (Roche Diagnostics) and (iv) all enzymes combined. An aliquot serving as negative control where no enzyme had been added was treated under identical conditions. The supernatants were removed and proteins were precipitated using trichloroacetic acid as described previously (Beinke et al., 1998). Samples were analysed by 7-5 % SDS-PAGE and Western blotting.

**Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment of intact HeLa cells.** Surface-biotinylated HeLa cells, still attached to the Petri dish, were treated with PI-PLC (1 U ml⁻¹; Roche Diagnostics) in DMEM for 1 h at 37 °C and 10 % CO₂. The supernatant was saved and cells were washed once with D-PBS before lysis. This was followed by co-immunoprecipitation with AIDA-I or anti-CD29/55 mAbs, and the subsequent analysis was by Western blotting.

**RESULTS**

**Purification of AIDA-I and secondary structure analysis**

For the identification of a potential cellular receptor(s) and for secondary structure analysis of the adhesin, functional AIDA-I protein was purified after mild heat extraction of intact *E. coli* 2787 or recombinant C600(pIB264) bacteria. Contaminating outer-membrane proteins, e.g. OmpA, OmpF or the AIDA c translocator, were removed by ultracentrifugation taking advantage of micelle formation between lipopolysaccharides and outer-membrane proteins (Fig. 1a, lane 4). Further purification was achieved by gel filtration on a Sephacryl S300 HR column. Although under denaturing conditions in SDS-PAGE the adhesin AIDA-I migrates with a molecular mass of about 100 kDa (Fig. 1a; columns 1–3, 6; see Benz & Schmidt, 1992b), the elution volume corresponded to proteins with molecular masses of around 450–600 kDa (Fig. 1b), indicating a potential oligomerization of AIDA-I to a pentamer or hexamer under these conditions. Fractions that by SDS-PAGE contained pure AIDA-I (Fig. 1a) were pooled.
concentrated by reverse PEG dialysis and used for all subsequent experiments. With this purification protocol a typical yield of pure AIDA-I in the range of 0.9–2.8 mg protein could be obtained from a 500 ml overnight culture.

To assess whether the conformation of the adhesin AIDA-I was maintained in the purified protein, we used sequence-based secondary-structure predictions in comparison with CD spectroscopy. Predictions using the GORA IV and SOPMA algorithms (Garnier et al., 1996; Geourjon & Deleage, 1995) indicated that extended β-strands might represent the dominant secondary structure of AIDA-I. This prediction corresponded very well with the evaluation of the CD spectra recorded with the purified adhesin (Fig. 1c). The characteristic minimum at 217 nm and the decline below 200 nm are in favour of β-strands as the major structural motif of the adhesin AIDA-I (approx. 10% α-helices, 46% β-strands and 42% random coil). Thus, during the isolation and purification steps the isolated protein most likely retained its native conformation. In addition, using the epithelioid HeLa cells as a model system in tissue culture, we confirmed that the purified adhesin had maintained its binding capacity (see below).

**Distribution of receptor molecules for the adhesin AIDA-I on mammalian cells**

The distribution of receptor molecules for AIDA-I on the cell surface was analysed by single or double immunofluorescence microscopy of fixed HeLa cells incubated with AIDA-I and/or with mAbs directed against β1-integrins (CD29) or decay-accelerating factor (CD55). These antigens had been shown to serve as receptors for bacterial adhesins (invasin of *Y. pseudotuberculosis* and Dr adhesins of *E. coli*) and thus served as exemplary controls for protein receptors. On fixed cells we observed regularly dispersed patches of bound AIDA-I (Fig. 2a) reminiscent of the staining pattern found for CD55 (Fig. 2c) but distinct from the homogeneous distribution of β1-integrins in the plasma membrane (Fig. 2b).

However, upon incubation of AIDA-I with viable cells, clustering of cellular receptors on the cell surface was observed (Fig. 2f1, g1). By contrast, the homogeneous distributions of β1-integrins (Fig. 2b, f2, g2) and CD55 (Fig. 2c) were largely unaffected. Thus, β1-integrins and the decay-accelerating factor (CD55) could be excluded as possible receptor molecules. To analyse whether the clustered

**Fig. 2.** Immunofluorescence detection of bound AIDA-I, β1-integrins and CD55 on HeLa cells. Purified AIDA-I was incubated with HeLa cells following (a–e) or prior to (f, g) fixation and subsequently probed with specific antiserum and Texas-red-conjugated secondary antibody (a, d, e, f1, g1). β1-Integrins (b, f2, g2) and CD55 (c) were detected with the respective mAbs and DTAF-conjugated secondary antibody. The antigen distribution was analysed by conventional (a–c) and high-resolution confocal laser scanning microscopy (pinhole 20 nm) showing apical (d, f) and median optical sections (e, g). Bars, 10 μm.
protein might be taken up by receptor-mediated endocytosis, high-resolution confocal laser scanning microscopy with or without membrane permeabilization was performed to distinguish between internal and external AIDA-I. We could not observe any differences in the immunofluorescence pattern, which indicates that although binding of AIDA-I to receptor molecules induces ligand–receptor clustering, bound adhesin seems not to be taken up under these conditions. Similar staining patterns were observed with other mammalian cell lines derived from various tissues of several species, e.g. with human intestinal epithelial cells (INT-407, undifferentiated Caco-2), monkey fibroblast-like cells (CV-1) and Chinese hamster ovary cells (CHO-K1). This indicated that the antigen recognized by AIDA-I is not restricted to human epithelial cells but is also expressed on cell lines derived from various tissues and species.

**Specific recognition of a cell-surface protein by AIDA-I**

In an effort to define the binding properties of the AIDA autotransporter adhesin with epithelial (HeLa) cells in more detail, we developed a cell-based ELISA. As shown in Fig. 3(a), binding of purified AIDA-I at a concentration of 80 nM reached equilibrium (95%) after 70 min. Based on these data, protein binding at equilibrium by using a series of AIDA-I concentrations (0.04–250 nM) was quantified (Fig. 3b). Saturation was achieved at a concentration of approximately 500 nM. The equilibrium dissociation constant was calculated to be around 2 nM which indicates a specific and high-affinity binding.

To address the biochemical nature of the potential host receptor, fixed HeLa cells were treated either with proteinase K to remove/disrupt surface-exposed proteins or with periodate to oxidize and modify susceptible carbohydrates in glycolipids and/or glycoproteins. AIDA-I bound efficiently to fixed untreated HeLa cells but exhibited only less than 20% binding to proteinase-K-treated monolayers (Fig. 4a), which suggested that the adhesin AIDA-I interacts with a surface-exposed protein and not with a glycolipid. This was further supported by experiments using solid-phase binding assays with extracted membrane glycolipids (data not shown). As expected, controls with cholera toxin recognizing the GM1 glycolipid demonstrated that binding to glycolipids is not affected by protease treatment. Interestingly, protease treatment apparently improved the recognition of CD29 by the anti-CD29 mAb (Fig. 4a, b).

To investigate whether AIDA-I recognizes a carbohydrate motif or might bind to a protein epitope, we examined the effect of periodate oxidation. Mild periodate oxidation at acidic pH has been shown to preferentially cleave vicinal

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**Fig. 3.** Time-course and binding characteristic of AIDA-I to HeLa cells by cell-surface ELISA. HeLa cells were grown in 96-well plates and, after fixation, incubated with purified AIDA-I protein (a, 80 nM; b, 0.04–250 nM). After several washing steps, bound AIDA-I was detected by specific anti-AIDA-I antibodies and HRP-coupled secondary antibody as described in Methods. (a) The calculated maximal binding ($B_{max}$) is indicated as a broken line. (b) The results of two independent experiments are shown (■, Expt 1; ○, Expt 2).
Fig. 4. Influence of protease treatment and periodate oxidation of HeLa cell monolayers on AIDA-I binding. Confluent monolayers of HeLa cells grown in 96-well plates were fixed. Binding of 5 μg AIDA-I ml⁻¹ and of control proteins was measured after pre-treatment of monolayers with buffer (a, b, solid columns), proteinase K (a, open column) or periodate oxidation (b, open column). Bound AIDA-I, cholera toxin (CT), CD29-specific mAb and biotinylated lectins (MAA, *Maackia amurensis*; ConA, *Concanavalin A*; STA, *Solanum tuberosum*) were analysed in a cell-surface ELISA with HRP-conjugated secondary reagents (expressed as a percentage of the buffer-treated control). Data are depicted as means of triplicate determinations; SD values of a typical experiment are also shown.

hydroxyl groups without affecting the structure of the polypeptide chain (Falk *et al.*, 1994; Woodward *et al.*, 1985). As shown in Fig. 4(b), AIDA-I binding was only moderately reduced (to 56 %) by this treatment, which suggests that the binding site could possibly consist of a proteinaceous and a carbohydrate part or alternatively only of a carbohydrate moiety of a glycoprotein, which due to its specific linkage pattern remains largely unaffected by oxidation. This possibility is emphasized by the *Solanum tuberosum* agglutinin (STA) recognizing N-acetylgalactose residues which under the conditions employed are apparently not modified by periodate oxidation. Concanavalin A binding is only reduced to 52 % following periodate oxidation which might be attributed to residual binding to non-oxidized galactose and N-acetylgalactose residues. By contrast, if the recognized motif is destroyed, the binding is completely abolished (e.g. cholera toxin, *Maackia amurensis*). Interestingly, the oxidation of carbohydrate moieties sometimes also increases recognition of protein epitopes as demonstrated for anti-CD29 mAbs used as controls. To analyse further whether AIDA-I might recognize a carbohydrate structure on the target-cell surface, different lectins (see Methods) were investigated for their capacity to interfere with adhesin binding using a 10-fold excess of the respective lectins. However, though all of the lectins bound to the cellular surface, binding of AIDA-I to the cell surface could not be reduced, so it seems likely that AIDA-I recognizes a proteinaceous structure.

**Co-immunoprecipitation of a surface-exposed glycoprotein with AIDA-I**

For the identification and isolation of the putative receptor from solubilized cellular extracts, HeLa cells were surface-biotinylated by incubation with the membrane-impermeable, charged biotin derivative N-hydroxysulfo-succinimide-biotin. The biotinylation of HeLa-cell surfaces did not affect the binding of AIDA-I, as shown by immunofluorescence-labelling studies (data not shown). Therefore, co-immunoprecipitation experiments with or without AIDA-I were performed using lysates of surface-biotinylated HeLa cells as described in Methods. To reduce non-specific protein binding, detergents (e.g. Triton X-100, Triton X-114, Nonidet NP-40, octylglucoside and CHAPS) in concentrations ranging from 0·08 to 3 % were investigated for their influence on the AIDA-binding activity. The strongest and most specific signals were reproducibly obtained by solubilization with 2 % CHAPS. Only by co-immunoprecipitation with AIDA-I in combination with the specific anti-AIDA-I antiserum, but not with pre-immune serum, could a protein of approximately 119 kDa be precipitated from biotinylated cell extracts (Fig. 5a). This clearly indicated that this surface-exposed cellular protein is specifically recognized and bound by AIDA-I. These findings further support the results of the biochemical characterization of the putative receptor moiety in the cell-surface ELISA studies. As the slightly diffuse migration pattern in SDS-PAGE is a characteristic property of glycoproteins, this indicated the putative AIDA-I receptor to be glycosylated and thus it was tentatively denoted gp119. Furthermore, gp119 could only be precipitated after detergent-mediated lysis of biotinylated cell membranes and although proteins contained in the tissue culture medium and in the cellular supernatant could also be strongly biotinylated, no other protein was reproducibly co-immunoprecipitated following incubation with AIDA-I (Fig. 5b).

Subsequently, the co-immunoprecipitated gp119 was subjected to two-dimensional electrophoresis using pre-cast pH-gradient gels between pH 3–10 and 4–7 as described in Methods. Isoelectric focusing indicated the pI of the gp119 be precipitated from biotinylated cell extracts. For the identification and isolation of the putative receptor from solubilized cellular extracts, HeLa cells were surface-biotinylated by incubation with the membrane-impermeable, charged biotin derivative N-hydroxysulfo-succinimide-biotin. The biotinylation of HeLa-cell surfaces did not affect the binding of AIDA-I, as shown by immunofluorescence-labelling studies (data not shown). Therefore, co-immunoprecipitation experiments with or without AIDA-I were performed using lysates of surface-biotinylated HeLa cells as described in Methods. To reduce non-specific protein binding, detergents (e.g. Triton X-100, Triton X-114, Nonidet NP-40, octylglucoside and CHAPS) in concentrations ranging from 0·08 to 3 % were investigated for their influence on the AIDA-binding activity. The strongest and most specific signals were reproducibly obtained by solubilization with 2 % CHAPS. Only by co-immunoprecipitation with AIDA-I in combination with the specific anti-AIDA-I antiserum, but not with pre-immune serum, could a protein of approximately 119 kDa be precipitated from biotinylated cell extracts (Fig. 5a). This clearly indicated that this surface-exposed cellular protein is specifically recognized and bound by AIDA-I. These findings further support the results of the biochemical characterization of the putative receptor moiety in the cell-surface ELISA studies. As the slightly diffuse migration pattern in SDS-PAGE is a characteristic property of glycoproteins, this indicated the putative AIDA-I receptor to be glycosylated and thus it was tentatively denoted gp119. Furthermore, gp119 could only be precipitated after detergent-mediated lysis of biotinylated cell membranes and although proteins contained in the tissue culture medium and in the cellular supernatant could also be strongly biotinylated, no other protein was reproducibly co-immunoprecipitated following incubation with AIDA-I (Fig. 5b).

Subsequently, the co-immunoprecipitated gp119 was subjected to two-dimensional electrophoresis using pre-cast pH-gradient gels between pH 3–10 and 4–7 as described in Methods. Isoelectric focusing indicated the pI of the gp119 protein, which migrated as a condensed single spot, to be around 5·2.
The AIDA-I receptor gp119 is an \(N\)-glycosylated integral membrane protein

To validate the assumption of the gp119 AIDA receptor representing a glycoprotein, the co-immunoprecipitates were subjected to enzymatic treatment with different glycosidases. As shown in Fig. 6, treatment with peptide-\(N\)-glycosidase F (Endo F) reduced the apparent molecular mass by 10–20 kDa to 106 kDa. However, treatment with sialidase, \(O\)-glycosidase or further degradation by the combination of all three enzymes had no effect on the mobility in SDS-PAGE. The activity of the enzymes was monitored by incubation with the immunoprecipitated CD29 and CD55 proteins where the expected molecular mass shifts due to the de-glycosylation were observed (data not shown). This experiment showed that the receptor of AIDA-I is an \(N\)-glycosylated as well as a surface-exposed protein, but that it probably does not contain terminal sialic acids or \(O\)-linked carbohydrates.

To elucidate whether gp119 might represent an integral membrane protein, a membrane-associated protein or a glycosylphosphatidylinositol (GPI)-anchored protein, further experiments were conducted. Attempts to dissociate gp119 from isolated cell membranes by treatment with high salt (1 M, 2 M NaCl in 50 mM Tris/HCl, pH 7-5) and basic pH (carbonate buffer pH 11-5) according to Fujiki et al. (1982) with subsequent subcellular fractionation were not successful (Fig. 7). By co-immunoprecipitation, gp119 was exclusively identified in the membrane fraction, indicating the

Fig. 6. Sensitivity of gp119 to treatment with glycosidases. HeLa cells were surface-biotinylated and lysates were subjected to co-immunoprecipitation with AIDA-I. Co-immunoprecipitates were boiled and left either untreated or treated with endo-glycosidase F, \(O\)-glycosidase, sialidase or all enzymes combined. After the enzyme treatment, proteins were separated by SDS-PAGE (7-5%) and analysed by affinity blotting with streptavidin–HRP. Molecular mass markers (in kDa) are given on the left.
putative AIDA-I-binding protein to represent an integral membrane protein. To address a potential membrane anchoring via a GPI-anchor, the selective release from the plasma membrane by digestion with bacterial PI-PLC was investigated. As demonstrated in Fig. 7, the GPI-anchored CD55 was cleaved by this treatment, whereas membrane-spanning integral membrane proteins like CD29 remained unaffected. To determine whether the AIDA-I receptor might potentially be linked via a GPI-anchor, intact biotinylated HeLa cells were treated with PI-PLC. Cell lysates and supernatants of PI-PLC-treated and untreated cells were subjected to AIDA co-immunoprecipitation and subsequently analysed by Western blotting. As shown in Fig. 7, gp119 could not be released by treatment with PI-PLC. Thus, the AIDA-I receptor protein most likely represents an integral membrane protein anchored to the plasma membrane via a membrane-spanning segment(s).

Database searches for AIDA-I receptor candidates

To identify potential candidates for the AIDA-I receptor protein gp119, we performed extensive database and literature searches (e.g. SWISS-PROT) based on properties such as a pI around 5.2, molecular mass around 119 kDa (as this value might not reflect the true molecular mass), integral membrane protein and N-glycosylation around 10–20 kDa. Although proteins with matching properties could not be found, we identified several membrane proteins which at least resembled the AIDA-I receptor protein in one or other of the biochemical characteristics. Potential candidates included αv-integrin (CD49f), αv-integrin (CD51), β1-integrin (CD29), ICAM-3 (CD50) and cadherins. However, specific monoclonal or polyclonal antibodies directed against these antigens did not recognize the co-immunoprecipitated AIDA-I receptor protein in Western blotting experiments.

DISCUSSION

The AIDA autotransporter/adhesion system belongs to the fast-growing family of autotransporter proteins that are used by a range of pathogenic Gram-negative bacteria either to transport virulence factors across the bacterial cell envelope to the surface or to even release these factors into the extracellular environment. All autotransporters described thus far are characterized by a common translocation mechanism. This is supported by homologies in the primary amino acid sequence and particularly accentuated by the common secondary structure of the C-terminal ‘translocator’ also described as the β-domain. In contrast, the translocated N-terminal α-domains (or ‘passenger’ proteins) of the different autotransporter molecules vary widely in sequence and function (Henderson et al., 1998, 2000; Henderson & Nataro, 2001; Loveless & Saier, 1997). The prototype of the autotransporter protein family is represented by the IgA protease of Neisseria gonorrhoeae which specifically cleaves the hinge region of S-IgA1 (Pohlner et al., 1987). Some of the more recently characterized autotransporter systems exhibit adhesin activities, indicating that they might be involved in the very first steps of microbial pathogenesis (Benz & Schmidt, 1989; Charles et al., 1989; Henderson & Owen, 1999; Stathopoulos et al., 1999). The strategy of surface presentation by autotransporter translocation is particularly intriguing as the available evidence points to a non-catalytic transport of the passenger proteins. Furthermore, to the best of our knowledge, a cellular receptor or binding protein on target cells recognized by an autotransporter/adhesin has not yet been elucidated.

In this study, we investigated the interaction of the purified AIDA autotransporter/adhesin (α-domain) with several epithelioid cell lines in tissue culture. Using HeLa cells as a model system, the binding was found to be specific to a high-affinity class of receptor molecules. By co-immunoprecipitation we could identify and further
characterize a glycoprotein (gp119) as an apparently novel integral membrane protein serving as the AIDA-I receptor (or 'AIDAR') on mammalian cells.

As fimbrial or afimbrial adhesins often exhibit lectin-like activities in recognizing carbohydrate structures as part of glycolipid or glycoprotein moieties (e.g. type 1 or P-pili), we were interested to see whether the AIDA-I receptor activity on the target-cell surface might also be associated with a carbohydrate structure or whether it might involve a peptide backbone. As most carbohydrate structures would be altered or even destroyed by periodate oxidation, intact cells were either treated with periodate or, alternatively, with proteases to remove proteinaceous antigens. As demonstrated in Fig. 4(a), protease treatment reduced binding of the AIDA-I adhesin to HeLa cell monolayers to less than 20%, while the binding of cholera toxin to G\textsubscript{M1} was not affected. By contrast, periodate oxidation reduced the binding of AIDA-I in this assay only moderately (Fig. 4b), while the binding of cholera toxin as well as that of several lectins was severely affected. That periodate oxidation did not completely abolish the binding of ConA and affected STA binding only marginally is probably due to the heterogeneity of the carbohydrate structures involved which might also include 3-linked glycosides that are not susceptible to periodate oxidation. Taken together, these results clearly indicate that the adhesin AIDA-I recognizes a proteinaceous antigen on the surface of HeLa cell monolayers and does not bind to a glycolipid. However, the contribution of carbohydrates (in particular in the form of glycoproteins) in the adhesin–receptor interaction could not be excluded.

Thus, as a next step we sought to isolate and identify the AIDA-I protein receptor by co-immunoprecipitation after solubilizing the binding activity from the HeLa cell membrane. To modify only surface-exposed proteins and to avoid accidental labelling of cytosolic proteins, biotinylation was performed employing a membrane-impermeable biotin derivat. As shown by immunofluorescence, biotinylation did not affect the binding of AIDA-I to HeLa cell monolayers. To largely maintain AIDA-I-binding activity during solubilization and to reduce non-specific binding during isolation, we investigated the suitability of several detergents (e.g. TX-100, TX-114, Nonidet NP-40, octyl-glucoside or CHAPS) in various concentrations. Consistent results could only be obtained by solubilization with 2% CHAPS, which not only solubilized the AIDA-I receptor protein but also preserved its binding activity. After solubilization of surface-biotinylated HeLa cells and co-immunoprecipitation, the precipitated proteins were analysed by one- and two-dimensional gel electrophoreses followed by Western blotting. Only in those cell-derived fractions that had been incubated with AIDA-I and with anti-AIDA-antiserum as well could a biotinylated protein be detected after co-immunoprecipitation (Fig. 5). This is strong evidence that the co-immunoprecipitation is specific, that the precipitated protein of about 119 kDa is a HeLa cell-surface protein and that this protein represents the AIDA-I receptor.

As the treatment of HeLa cells with periodate nevertheless had a slight effect on AIDA-I binding, we were interested to determine whether the 119 kDa protein receptor might be glycosylated. Thus, the immunoprecipitated protein was subjected to treatment with several glycosidases. As demonstrated in Fig. 6, only the incubation with N-glycosidase had an effect on the migration of the AIDA-I receptor in SDS-PAGE by reducing the apparent molecular mass to about 106 kDa, while incubation with O-glycosidase and sialidase had no noticeable effects. This suggests that the 119 kDa protein is N-glycosylated and that the AIDA-I receptor represents a membrane glycoprotein (gp119).

To further analyse whether gp119 is a peripheral or an integral membrane protein or whether it might be anchored by a GPI-anchor, extraction experiments were performed. As the gp119 AIDA-I receptor could not be extracted from HeLa cell membranes using different extraction conditions (salt concentrations, pH) (Fig. 7a), a mere association of the AIDA-I receptor with the cellular membrane could be excluded. Furthermore, upon treatment with the GPI-anchor-specific phospholipase (PI-PLC) using the membrane proteins CD55 (GPI-anchored protein) and CD29 (integral-membrane protein) as controls to monitor PI-PLC efficiency, we could also exclude the presence of a GPI-anchor in gp119. This was further supported by results from immunofluorescence experiments (data not shown). Thus, AIDA-I recognizes an integral N-glycosylated membrane glycoprotein of 119 kDa in HeLa cells which we tentatively termed ‘AIDAR’. Employing cell lines originating from different tissues and species as a model system, we found that the AIDA-I receptor seems to be expressed on various cell types as has been shown for other bacterial adhesion systems (e.g. type 1 or P pili, Hia) (Klemm, 1994; St Geme et al., 1996). How this might potentially influence tissue tropism remains to be elucidated.

These findings, together with the observation of ligand-induced receptor clustering, prompted us to investigate the potential induction of signalling events and a putative uptake of bound AIDA-I. Signalling events as well as uptake have been reported to be mediated by several bacterial factors, e.g. the P-adhesins in combination with uroepithelial cells (Hedlund et al., 1996, 1999), for the invasin protein of \textit{Y. pseudotuberculosis} recognizing \(\beta_1\)-integrins (Isberg & Leong, 1990) and for the internalin A protein of \textit{L. monocytogenes} using E-cadherin as yet another ‘pirated’ cellular adhesion molecule (Mengaud et al., 1996). As specific phosphorylation is often involved in signal transduction, we investigated AIDA-I-mediated changes in phosphorylation. However, in both tissue culture models (HeLa, CaCo-2) investigated, neither protein phosphorylation nor internalization of bound AIDA-I could be detected (data not shown). This might indicate...
that the interaction of AIDA-I with its receptor protein is primarily involved in the initial adherence step of diarrhoeagenic *E. coli* to epithelial cells.

By screening databases for proteins with matching biochemical characteristics found for the AIDA-I receptor protein (gp119, integral surface protein, pI ~ 5.2), we could not identify a candidate receptor protein. Although very few potential candidate antigens matched at least one of the criteria, we nevertheless probed the AIDA-I co-immunoprecipitates by immunoblotting with antibodies directed against cell-surface antigens (e.g. α₅- and α₆-integrin, ICAM-3, cadherins, gp130) selected by this procedure. However, none of the specific antibodies to potential candidates recognized the AIDA-I receptor protein.

In conclusion, in this study we have identified the receptor protein for the AIDA autotransporter/adesin of diffusely adhering *E. coli*, tentatively termed ‘AIDAR’, as an apparently novel, N-glycosylated integral membrane protein of 119 kDa which lacks a GPI-anchor. Studies to clone and further functionally characterize the AIDA-I receptor are under way in our laboratory.

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