Structure−Function Analysis of the Adherence-Binding Domain on the Pilin of *Pseudomonas aeruginosa* Strains PAK and KB7†

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ABSTRACT: The pili of *Pseudomonas aeruginosa* mediate bacterial binding to human epithelial cell surfaces. We have previously shown that a 17-residue synthetic peptide, KCTSDQDEQFIPKGCSK, corresponding to the C-terminal sequence of the PAK pilin protein (residues 128−144) contains the adherence binding domain. Another pilin strain, KB7, has been cloned and sequenced [Paranchych et al. (1990) in *Pseudomonas Biotransformations, Pathogenesis and Evolving Biotechnology*, pp 343−351, American Society for Microbiology, Washington, DC]. The C-terminal 17-residue sequence of the KB7 pilin is SCATTVDAKFRPNGCTD, which is semiconserved as compared to the PAK sequence. In this study, the interactions between the A549 human lung carcinoma cells and the two *P. aeruginosa* pilin strains were elucidated using a single alanine replacement analysis on the C-terminal 17-residue synthetic peptide of the pilins. The ability of these peptide analogs to inhibit the binding of the biotinylated PAK pili to A549 cells was assessed. Six PAK amino acid side chains (Ser^{131}, Gln^{136}, Ile^{138}, Pro^{139}, Gly^{141}, and Lys^{144}) and nine KB7 side chains (Ala^{130}, Thr^{131}, Thr^{132}, Val^{133}, Asp^{134}, Ala^{135}, Lys^{136}, Arg^{138}, and Pro^{139}) were found to be important in mediating the pilus adhesin binding to A549 cells. In addition, a flexible peptide analog with both cysteine residues replaced by alanine failed to inhibit the binding of PAK pili to A549 cells. This suggests that the interactions between the pilin ligand and the A549 cell surface receptors are dependent on the conformation mediated by the disulfide bridge (Cys^{129} and Cys^{142}). The residues considered to contribute to bacterial adherence are referred to as the "adhesintope". Four PAK and three KB7 side chains were located in a structurally more rigid region of the disulfide-bridged peptide as revealed by two-dimensional NMR studies [McInnes et al. (1993) *Biochemistry 32*, 13432−13440]. The structural aspects of the pilin−receptor interactions related to the mapped adhesintope sequences are discussed. The dissimilarities between the PAK and KB7 adhesintopes may suggest that compensatory mutations could occur among different pilin strains so as to allow the pilin adhesins to interact with the same receptor.

Adherence is a critical initial event of pathogenesis (Beachey, 1981). The opportunistic pathogen *Pseudomonas aeruginosa* employs several distinct adhesins to mediate attachment to host mucosal epithelial cells (Prince, 1992; Irvin, 1993), including pili (Woods et al., 1980; Doig et al., 1988), alginate (Ramphal & Pier, 1985; Doig et al., 1987), exoenzyme S (Baker et al., 1991; Lingwood et al., 1991), and outer membrane proteins (Saiman et al., 1990; Ramphal et al., 1991b). Among these adhesins, the pilus plays an unique role in the adherence process and is believed to be responsible for the initial attachment of the microorganism to its host (Pier, 1985; Irvin, 1993). Woods et al. (1980) were the first to suggest the importance of pili to *P. aeruginosa* adherence in the upper respiratory tract. During the past decade, investigations on the role of *P. aeruginosa* pili in interacting with different cell types such as buccal epithelial cells (Doig et al., 1988), tracheal epithelial cells (Ramphal et al., 1984), epidermal cells (Sato & Okinaga, 1987), corneal epithelial cells (Rudner et al., 1992), and A549 pneumocyte cells (Chi et al., 1991) have further illustrated the importance of pilus adhesin in mediating the adherence process.

The *P. aeruginosa* pili are polarly distributed and are composed of a 13−17 kDa protein subunit termed pilin (Pasloske et al., 1988; Paranchych et al., 1990). More than eight pilin genes have been described (Paranchych et al., 1990), of which only a single copy of the pilin structural gene is encoded in the bacterial chromosome. The amino acid sequence of one of the best studied pilin strains, the PAK pilin, has 144 amino acid residues (Sastry et al., 1985) that contains an adherence binding domain at its C-terminus (Paranchych et al., 1985; Irvin et al., 1989). This region contains a consensus intrachain disulfide bridge formed by Cys^{129} and Cys^{142}. The C-terminal region of the PAK pilin is only exposed at the tip of the pilus where receptor binding occurs (Lee et al., 1994). Synthetic peptides containing the C-terminal domain competitively inhibit pilus binding to human epithelial cells (Irvin et al., 1989).

Monoclonal antibodies specific for the C-terminal domain of *P. aeruginosa* PAK pilin have been produced (Doig et al., 1990). One of these antibodies, PK99H, recognizes the

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C-terminal adherence binding domain on the PAK pilin and effectively inhibits *P. aeruginosa* binding to epithelial cells (Doig et al., 1990). This monoclonal antibody also provided passive immunity against a homologous strain in challenge studies in mice (Sheth et al., 1995). Previously, the antigenic epitope recognized by PK99H was determined using a series of single alanine-substituted synthetic peptide analogs corresponding to the C-terminal 17-residue sequence of the PAK pilin (Wong et al., 1992). The PK99H epitope was found to consist of the linear sequence DEQFIPK from residue 134 to residue 140, which was subsequently confirmed by utilizing a combinatorial peptide library (Wong et al., 1994).

As the C-terminal domain of *P. aeruginosa* pilin plays a significant role in infection, it was of interest to establish the structure−activity relationship of the binding domain of the pilus adhesin. At this point, very little is known about the *P. aeruginosa* pilus adhesin−receptor interaction. In this study, a similar approach to epitope mapping was applied to identify amino acid residues that contribute significantly to adhesin function (referred to as the "adhesintope") on the C-terminal region of the PAK pilin to A549 human pneumocyte cells. Recently, the NMR solution structures of the C-terminal 17-residue peptides of *P. aeruginosa* pilin strains PAK (McInnes et al., 1993) and KB7 (Campbell et al., 1995) have been derived. This provides significant structural information and sheds light on how the pilin peptide interacts with its antibody as well as its receptor. In this report, the pilin adhesintope residues in relation to available NMR structural conformation are discussed.

MATERIALS AND METHODS

Bacterial Pili. The bacterial pili employed in this study were obtained from the *P. aeruginosa* strain PAK/2pfs. Purification of pili was as previously described (Paranchych et al., 1979).

Peptide Synthesis. The amino acid sequences of the C-terminal 17-residue peptides of the PAK pilin and KB7 pilin are Ac-KCTSDQDEQFIPKGCSK-OH and Ac-SCATTVDAKFRPNGCTD-OH, respectively, where Ac denotes an N^{α}-acetylated peptide and OH indicates a peptide with a free α-carboxyl group. These two peptides are referred to as PAK(128−144)$_{ox}$ and KB7(128−144)$_{ox}$ in this report, where ox denotes an oxidized peptide with an intrachain disulfide bond between Cys^{129} and Cys^{142}. The peptide analogs were named according to the pilin strain, the residues being substituted as well as their corresponding positions. For example, PAK(P139A) denotes a PAK pilin peptide analog with Pro^{139} substituted by alanine.

Peptide syntheses were performed following the general procedure for solid-phase synthesis as described by Erickson and Merrifield (1976) on an Applied Biosystems 430A solid-phase peptide synthesizer (Foster City, CA). All amino acids used were protected at the α-amino position with the *tert*-butyloxycarbonyl (Boc)[1] group (Bachem Inc., Philadelphia, PA). Syntheses of peptides with a C-terminal lysine and a free α-carboxyl group were started with Boc-lysine(2-ClZ)-OCH_2-Pam resin (Applied Biosystems; 1% cross-linked, 0.67 mmol of Lys/g). The synthesis of C-terminal alanine peptide analogs was initiated by esterification of the cesium salt of Boc-alanine to copoly(styrene, 1% divinylbenzene)chloromethyl resin (Pierce Chemical Co.; 0.9 mmol of amino groups/g). In addition, synthesis of the peptide amide was carried out using copoly(styrene, 1% divinylbenzene)-benzhydrylamine hydrochloride resin at a substitution of 0.92 mmol of amino groups/g of resin (Bachem Inc., Philadelphia, PA). All amino acids were double coupled using dicyclohexylcarbodiimide (DCC) generated symmetric anhydrides in *N,N*-dimethylformamide (DMF) for the first coupling and dichloromethane (DCM) for the second coupling. Final acetylation was performed on the instrument using acetic anhydride.

After synthesis, the peptides were cleaved from the resin support with anhydrous hydrogen fluoride (20 mL/g of peptide resin) in the presence of 10% (v/v) anisole and 1% (v/v) 1,2-ethanedithiol as scavenging reagents for 1 h at −4 °C using type 1B HF-reaction apparatus (Peninsula Laboratories Inc., Belmont, CA). The solvent mixture was then removed under reduced pressure. The resin was washed with anhydrous diethyl ether (3 × 25 mL), and peptide was extracted with neat acetic acid (3 × 25 mL). The peptide solution was diluted with distilled water and then lyophilized.

Purification of crude peptides was performed by reversed-phase HPLC on an Applied Biosystems 400 solvent delivery system and a 783A programmable absorbance detector connected to a Synchropak RP-4 (250 × 21.2 mm i.d.) reversed-phase column (Synchrom Inc., Lafayette, IN) using a linear AB gradient of 0.2% B/min at a flow rate of 5 mL/min, where solvent A was 0.05% trifluoroacetic acid (TFA)/water and solvent B was 0.05% TFA/acetonitrile. The absorbance was recorded at 210 nm. The purity and authenticity of the peptides were examined by means of analytical HPLC, amino acid analysis, and mass spectrometry. Analytical HPLC was done on an Aquapore RP-300, C_8 reversed-phase column (220 × 4.6 mm i.d., Brownlee Laboratories, Santa Clara, CA). Purified peptides were hydrolyzed with 6 N HCl with 0.1% (v/v) phenol in sealed, evacuated tubes at 110 °C for 22 h, and amino acid analyses were performed on a Beckman System 6300 high-performance automatic analyzer (Beckman, Palo Alto, CA). The molecular weight and purity of the peptides were confirmed with a BioIon 20 Plasma Desorption Time of Flight mass spectrometer (Uppsala, Sweden).

For those 17-residue peptides containing two cysteines in their sequence, air oxidation was performed by stirring a solution of the peptide (0.1 mg/mL) in 100 mM NH_4HCO_3, pH 8.2, overnight at room temperature. Completion of oxidation was examined by treating 100 μL of peptide solution with 10 μL of a 1 mg/mL aqueous solution of *N*-ethylmaleimide (NEM) (Lee et al., 1990). The HPLC chromatograms of NEM-treated and untreated peptides were compared. Only the reduced peptide with free cysteine residues could react with NEM, and the modified peptide eluted off the column with a retention time much later than that of the oxidized or reduced peptide (Lee et al., 1990).

[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; DCC, dicyclohexylcarbodiimide; 2-ClZ, 2-chlorobenzyloxycarbonyl; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; HBSS, Hanks' balanced salt solution; HOBT, *N*-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; I_{50}, concentration of peptide required to produce 50% inhibition in a competitive binding assay; NEM, *N*-ethylmaleimide; NMP, *N*-methylpyrrolidone; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid.

Ammonium bicarbonate in the peptide solution was first neutralized with acetic acid and subsequently removed by repeated lyophilization.

Biotinylation of PAK Pili. The PAK pili were biotinylated with a stock solution of 20 mg/mL biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma, St. Louis, MO) in dimethyl sulfoxide (DMSO). Limited biotinylation was attained by controlling the reaction time and by rapid removal of excess biotinylation reagent. The PAK pili (1 mL, 1.76 mg/mL) was pipetted into dialysis tubing (MW ~12 000 −14 000, 10 mm in diameter). A 30 μL aliquot of the stock biotinylation reagent was then added. The dialysis tubing was sealed and placed inside a 50 mL conical culture tube. The reaction was allowed to proceed for 45 min at room temperature under constant shaking in a gyroshaker. Glycine (10 mM) was added to quench the reaction, and excess biotin ester was removed by extensive dialysis with four changes of phosphate-buffered saline (PBS), pH 7.4 at 4 °C. The biological activities of the biotinylated PAK pili were checked by examining the ability of the biotinylated pili to bind to PAK pilus-specific monoclonal antibody PK99H (data not shown) as well as to A549 cells (Figure 1A).

Biotinylation of Synthetic Peptides. Biotinylation was performed by manual coupling of activated biotin to the N-terminus of the peptide. Before the biotinylation, four glycine residues were coupled to the N-terminus of the peptide to function as a spacer arm. Biotin (Sigma, St. Louis, MO) was activated by reaction with 1 equiv of 1 M *N*-hydroxybenzotriazole (HOBT) in *N*-methylpyrrolidone (NMP) and 1 equiv of 1 M DCC in NMP and stirred at room temperature for 30 min. The reaction mixture was then filtered through a sintered glass funnel. Peptide-resin after removal of the N-terminal protecting group was coupled with the activated biotin solution for 1 h with mixing. The reaction mixture was vortexed for 7 min with 700 μL of diisopropylethylamine (DIEA) added. The solution was drained and washed with NMP. The coupling efficiency was verified with a ninhydrin test. The biotinylated peptides were then cleaved from the resins, purified, oxidized, and verified according to the procedures described in the peptide synthesis section above. A negative control peptide, which corresponds to the sequence 75−84 of the PAK pilin protein, was synthesized and biotinylated. This biotinylated peptide has the sequence biotin-GGGGGVAADANKLG-amide.

Cell Culture. The human lung carcinoma A549 cell line ATCC CCL 185, Batch F-8669, was obtained from the American Type Culture Collection (Rockville, MD). This cell line was chosen in this study because it contains PAK pili-specific receptors (Chi et al., 1991) and features properties of type II alveolar epithelial cells (Lieber et al., 1976). Thus, it represents a useful human epithelial cell model for examining *P. aeruginosa* adherence. The cells were grown in a 25 cm^2 tissue culture flask (Corning, NY) in Waymouth MB 752/1 medium supplemented with 10% (v/v) fetal calf serum, 1 mM sodium pyruvate, and antibiotic−antimycotic solution (100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B). All tissue culture reagents were obtained from GIBCO-BRL (Life Technologies Inc., New York). The culture was maintained at 37 °C under an atmosphere of 5% CO_2 in a humidified incubator. The cells were passaged at 4−5-day intervals. The cell monolayer was detached by a 5-min incubation in trypsin−EDTA solution (0.05% w/v trypsin−0.53 mM EDTA in Ca^{2+}/Mg^{2+}-free PBS) at 37 °C. The harvested cells were then washed and centrifuged twice in plain medium, and the final pellet was resuspended in medium with supplements. Passages 82−87 were employed in this study.

Whole Cell Binding Assay. A549 cells were suspended in Waymouth medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 1 mM sodium pyruvate, and the antibiotic−antimycotic solution (assay medium). The cell density was adjusted to 4×10^5 cells/mL. A549 cells were seeded into a 96-well tissue culture plate (Corning, New York) by transferring an aliquot of 100 μL of the cell suspension to each well. The plate was incubated at 37 °C overnight. After the incubation, the medium in each well was removed manually by a multichannel pipet using pipet tips specialized for electrophoresis (Bio-Rad Laboratories Inc., Hercules, CA). The plate was washed once with 200 μL of assay medium per well. During each washing, the buffer was delivered slowly to the wells in order to minimize detachment of the cells. Biotinylated PAK pili (stock solution: 0.88 mg/mL, diluted 1:1000) were mixed with serially diluted competing ligands (the peptide analogs), and the assay mixtures were added to the wells (75 μL/well). In competitive binding assays where biotinylated peptides were employed, biotinylated peptide (2 μM) was mixed with serially diluted competing ligands. Sodium azide (0.02%) was added to the assay medium to prevent possible internalization of the ligands into the cells. Following a 2-h incubation at 37 °C, the plate was washed three times with 250 μL of assay medium per well and once with 250 μL of Hanks' balanced salt solution (HBSS) per well. The cell monolayers were then fixed with 100 μL of glutaraldehyde per well (0.25% in HBSS) and incubated at 37 °C for 1 h. The plate was then washed three times with HBSS, and the unreacted glutaraldehyde was neutralized with 250 μL of 50 mM glycine per well. Following an 1-h incubation at 37 °C, the plate was washed twice with 250 μL HBSS per well. Streptavidin-alkaline phosphatase (GIBCO-BRL, Life Technologies Inc., New York) was diluted 1:3000 in PBS containing 1% (w/v) BSA, and 75 μL of the solution was added to each well. The reaction was allowed to proceed at room temperature for 1 h. The plate was then washed four times with 200 μL of PBS per well containing 0.05% (w/v) BSA and once with 200 μL of 50 mM Tris-buffered saline, pH 7.5. Following the washes, 100 μL of *p*-nitrophenyl phosphate per well [1 mg/mL in 10% (v/v) aqueous diethanolamine, pH 9.8] and 0.1 mM levamisole were added, and the plate was incubated at room temperature. The absorbance at 405 nm was determined by a Titertek Multiskan Plus MK II microplate reader (Flow Laboratories Inc., McLean, VA).

RESULTS

Binding of PAK Pili and PAK Synthetic Peptide to A549 Cells. The C-terminal region of the PAK pilin has been shown to contain the adherence binding domain that binds to epithelial cells (Paranchych et al., 1985; Irvin et al., 1989). The adherence of *P. aeruginosa* to epithelial cells can be blocked either by peptides derived from the C-terminal sequence of the PAK pilin or by antibodies raised against this region (Irvin et al., 1989; Doig et al., 1990). In this study, the highly specific interaction between biotin and streptavidin was used as the reporter group to investigate the binding of PAK pili to A549 cells, a cell line which has

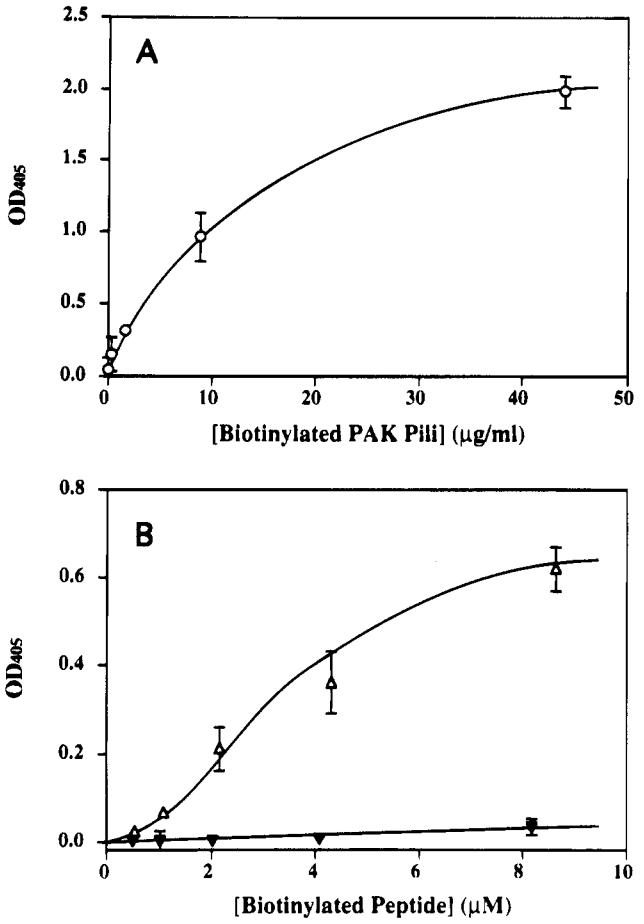

FIGURE 1: Direct binding of biotinylated PAK pili (O, panel A) and biotinylated PAK(128−144)$_{ox}$ peptide (△, panel B) to A549 cells. A biotinylated synthetic peptide corresponding to sequence 75−84 of the PAK pilin (▼, panel B) was used as a negative control. The amino acid sequences of the peptides can be found in Materials and Methods.

been shown to contain receptor sites specific for PAK pili (Chi et al., 1991). The biotinylated probes of both PAK pili and the C-terminal 17-residue synthetic peptide of the PAK pilin were made. The ability of these biotinylated probes to bind to A549 cells was determined. In Figure 1, both biotinylated PAK pili and peptide bind to A549 cells in a concentration-dependent manner, whereas a control biotinylated peptide shows no binding to A549 cells even at high concentration (Figure 1B). The signal obtained from the biotinylated PAK pili was considerably higher than that of the biotinylated peptide, suggesting that the PAK pilus may be biotinylated at more than one site, thus allowing for an amplification of the signal. Since the size distribution of the PAK pili is polydispersed, it is not possible to determine the degree of biotinylation of the pili in terms of molar unit, and so the value is reported as micrograms per milliliter.

Binding specificity between the A549 cells and the two biotinylated probes was also assessed. Figure 2 shows the result of a competitive binding assay; the binding of both biotinylated PAK pili and biotinylated PAK peptide to A549 cells can be inhibited specifically by the C-terminal PAK peptide, PAK(128−144)$_{ox}$ (Figure 2).

Adhesintope Mapping of the P. aeruginosa PAK Pilin. Residues on the PAK pilin that contributed to receptor binding (the adhesintope) were determined in a competitive binding assay employing a series of single alanine-substituted

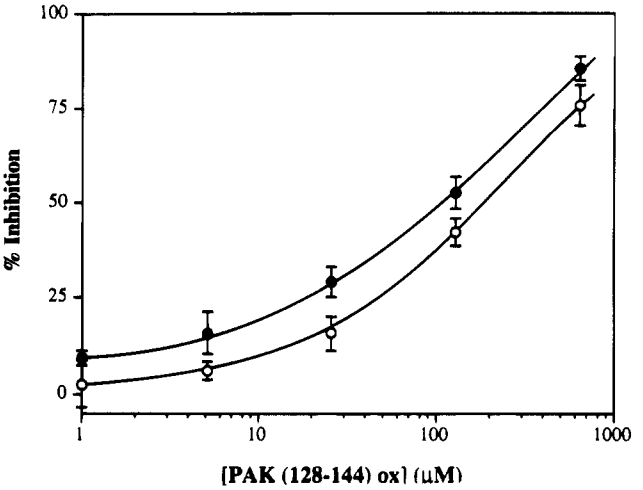

FIGURE 2: Competitive binding profiles showing the inhibitory effect of the synthetic peptide PAK(128−144)$_{ox}$ on the binding of the biotinylated PAK pili (O) and biotinylated PAK(128−144)$_{ox}$ peptide (●) to A549 cells.

(A) PAK Peptide Analogs

		128																144	
Native PAK	Ac	K	C	T	S	D	Q	D	E	Q	F	I	P	K	G	C	S	K	OH
PAK (K128A)	Ac	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	OH
PAK (T130A)	Ac	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	OH
PAK (S131A)	Ac	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	OH
PAK (D132A)	Ac	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	OH
PAK (Q133A)	Ac	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	OH
PAK (D134A)	Ac	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	OH
PAK (E135A)	Ac	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	OH
PAK (Q136A)	Ac	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	OH
PAK (F137A)	Ac	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	OH
PAK (I138A)	Ac	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	OH
PAK (P139A)	Ac	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	OH
PAK (K140A)	Ac	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	OH
PAK (G141A)	Ac	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	OH
PAK (S143A)	Ac	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	OH
PAK (K144A)	Ac	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	OH

(B) KB7 Peptide Analogs

		128																144	
Native KB7	Ac	S	C	A	T	T	V	D	A	K	F	R	P	N	G	C	T	D	OH
KB7 (S128A)	Ac	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	OH
KB7 (A130G)	Ac	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	OH
KB7 (T131A)	Ac	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	OH
KB7 (T132A)	Ac	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	OH
KB7 (V133A)	Ac	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	OH
KB7 (D134A)	Ac	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	OH
KB7 (A135G)	Ac	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	OH
KB7 (K136A)	Ac	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	OH
KB7 (F137A)	Ac	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	OH
KB7 (R138A)	Ac	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	OH
KB7 (P139A)	Ac	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	OH
KB7 (N140A)	Ac	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	OH
KB7 (G141A)	Ac	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	OH
KB7 (T143A)	Ac	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	OH
KB7 (D144A)	Ac	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	OH

FIGURE 3: Diagram showing the amino acid sequences of the single alanine-substituted peptide analogs of the PAK (panel A) and KB7 (panel B) C-terminal pilin peptides. The two alanine residues in the KB7 peptide are substituted by glycine. The boxed residues indicate identical residues in the PAK and KB7 sequence.

peptide analogs corresponding to the C-terminal 17-residue region of PAK pilin. Each residue (except the two cysteines) was substituted one at a time with an alanine along the 17-residue peptide sequence (Figure 3A). The ability of these peptide analogs to inhibit the binding of the biotinylated PAK pili to A549 cells was then determined. If the peptide analog shows a significant decrease in its ability to inhibit the binding of biotinylated PAK pili to A549 cells, the side chain substituted was considered to be important for receptor interaction and part of the adhesintope.

Table 1: Identification of the Residues That Contribute to Receptor Binding on the PAK and KB7 Pilin by Single Alanine or Glycine Replacement Analysis on the C-Terminal 17-Residue Peptide of Both Pilin Strains

PAK peptide analogs[a]	I_{50} (μM)[b]	KB7 peptide analogs[a]	I_{50} (μM)[b]
native	200 ± 23	native	190 ± 21
K128A	240 ± 25	S128A	110 ± 22
T130A	95 ± 26	**A130G**	**>800**
S131A	**>710**	**T131A**	**>650**
D132A	100 ± 21	**T132A**	**>780**
Q133A	260 ± 33	**V133A**	**>960**
D134A	70 ± 28	**D134A**	**>860**
E135A	430 ± 49	**A135G**	**630 ± 66**
Q136A	**>790**	**K136A**	**>970**
F137A	160 ± 19	F137A	140 ± 29
I138A	**>940**	**R138A**	**>980**
P139A	**>1000**	**P139A**	**>840**
K140A	250 ± 22	N140A	240 ± 45
G141A	**>1000**	G141A	350 ± 44
S143A	100 ± 17	T143A	160 ± 5
K144A	**>830**	D144A	470 ± 44

[a] The peptide analogs are labeled according to the residue being substituted (first letter), by alanine or glycine (last letter), and the sequence position (middle number). Native denotes the native 17-residue pilin peptides (see Materials and Methods). The regions in boldface denote important residues (I_{50} values greater than 3-fold of the native peptide) for A549 cell binding, i.e., the adhesintope. [b] Values represent the mean concentration of the peptide analogs required to inhibit biotinylated PAK pili binding to A549 cells by 50% ± standard deviation. Data are means of three triplicate assays.

The changes in the inhibitory effects of the alanine-substituted peptides were reflected by corresponding shifts of the binding curves. The relative importance of individual residues along the PAK 128−144 region that contributed to A549 cell binding is shown by their respective I_{50} values as deduced from the competitive binding curves. The I_{50} value is the concentration of peptide required to reduce the binding of biotinylated PAK pili to A549 cells by 50%. A summary of the I_{50} values of all oxidized PAK 17-residue peptide analogs is shown in Table 1. A comparison of the I_{50} values with respect to the native peptide revealed that six peptide analogs have I_{50} values greater than 3-fold that of the native peptide (I_{50} = 200 μM). The I_{50} values of these six peptide analogs are PAK(S131A), >710 μM; PAK(Q136A), >790 μM; PAK(I138A), >940 μM; PAK(P139A), >1000 μM; PAK(G141A), >1000 μM; and PAK(K144A), >830 μM (Table 1).

Importance of the Intrachain Disulfide Bridge. The two cysteine residues (residues 129 and 142) located at the C-terminal region of the *P. aeruginosa* pilin are highly conserved among different strains (Paranchych et al., 1990). Previously, the significance of the intrachain disulfide bridge has been addressed by comparing the direct binding of the oxidized and reduced peptides to buccal epithelial cells (Irvin et al., 1989). In this study, the importance of the disulfide bridge was further confirmed by synthesizing a PAK peptide analog with both cysteine residues substituted by alanine, and its ability to compete the binding of biotinylated PAK pili was examined. A much higher concentration of this peptide analog (>990 μM; Table 2) was required to inhibit the binding of biotinylated PAK pili to A549 cells as compared with the native peptide (200 μM). Thus, the disulfide bridge contributes significantly to the adhesin function of the PAK peptide, confirming our previous observations (Irvin et al., 1989).

Table 2: Examination of the Importance of the Disulfide Bond and the Isoleucine Residue for PAK Pilin Peptide Binding to A549 Cells

Peptide[a]	Sequence[b]	I_{50} (μM)[c]
	128 144	
PAK native	KCTSDQDEQFIPKGCSK	200
PAK (I138R)	KCTSDQDEQF**R**PKGCSK	>720
PAK (C129A, C142A)	K**A**TSDQDEQFIPKG**A**SK	>990

[a] PAK native is the synthetic peptide corresponding to the C-terminal sequence of the *P. aeruginosa* PAK pilin. See legend of Table 1 for peptide nomenclature. [b] The bold letters denote substituted amino acid residues. [c] Values indicate the mean concentration of the peptides required to inhibit the binding of biotinylated PAK pili to A549 cells by 50%.

The KB7 Pilin Adhesintope. The C-terminal region of the KB7 pilin shows some homology to that of the PAK strain (Figure 3). Six consensus residues are found in both peptide sequences; two of these residues are the highly conserved cysteine residues that form a disulfide bond. Interestingly, of these six homologous residues, four are found in the adhesintope sequence as deduced from the PAK peptide (Pro^{139}, Gly^{141}, Cys^{129}, and Cys^{144}; Tables 1 and 2). However, the remaining four residues found in the PAK adhesintope sequence (Ser^{131}, Gln^{136}, Ile^{138}, and Lys^{144}) are mutated to Thr^{131}, Lys^{136}, Arg^{138}, and Asp^{144}, respectively, in the KB7 sequence (Figure 3). In order to compare whether the KB7 pilin utilizes the same positional residues as those of the PAK pilin to bind to the A549 cell receptor, the KB7 pilin adhesintope was mapped, employing the same approach as that of the PAK pilin. The two alanine residues present on the KB7 sequence were substituted by glycine, whereas all other residues except the two cysteines were replaced by alanine (Figure 3B). The I_{50} values of these peptide analogs were then determined, and the results are shown in Table 1. Despite the heterogeneity in its amino acid sequence, the KB7 peptide was shown to be as effective as the PAK peptide in the inhibition of biotinylated PAK pili binding to A549 cells (I_{50} = 190 μM for KB7 peptide and 200 μM for PAK peptide; Table 1). However, the composition of the KB7 pilin adhesintope is quite different from that of the PAK strain. Nine KB7 peptide analogs were found to have I_{50} values greater than 3-fold that of the native peptide (I_{50} = 190 μM). These peptides are KB7(A130G), >800 μM; KB7(T131A), >650μM; KB7(T132A), >780 μM; KB7(V133A), >960 μM; KB7(D134A), >860 μM; KB7(A135G), 630 μM; KB7(K136A), >970 μM; KB7(R138A), >980 μM; and KB7(P139A), >840 μM (Table 1). The residues substituted in these peptide analogs are considered to be important residues that constitute the KB7 adhesintope.

DISCUSSION

During the past decade, extensive research has been undertaken to study pilus-mediated adherence of *P. aeruginosa* to human respiratory epithelial cells. Different receptor candidates have been reported for the pilus-specific interactions: for instance, glycosphingolipids such as asialo-GM_1 and asialo-GM_2, sialic acid containing glycosphingolipids and lactosylceramide (Krivan et al., 1988; Baker et al., 1990; Lee et al., 1994; Sheth et al., 1994), and

Table 3: Summary of Residues That Are Important for the Binding of both PAK and KB7 C-Terminal 17-Residue Pilin Peptides to A549 Cells

category	PAK pilin	KB7 pilin
polar	Ser^{131}, Gln^{136}	Thr^{131}, Thr^{132}
hydrophobic	Ile^{138}	Ala^{130}, Val^{133}, Ala^{135}
ionic	Lys^{144}	Asp^{134}, Lys^{136}, Arg^{138}
conformational control	Pro^{139}, Gly^{141}, Cys^{129}, Cys^{142}	Pro^{139}

glycoproteins (Doig et al., 1989; Irvin et al., 1989). The carbohydrate moieties of both glycoproteins and glycosphingolipids are believed to be involved in pilus binding. For example, pilus-specific disaccharides such as βGalNAc-(1−4)βGal on asialo-GM_1 and asialo-GM_2 (Krivan et al., 1988; Sheth et al., 1994), βGal(1−3)βGlcNAc, and βGal-(1−4)βGlcNAc (Ramphal et al., 1991a) have been reported. Receptor binding by the pilus is a tip-associated event where the adherence binding domain is located at the C-terminal side of the pilin which is exposed at the tip of the pilus protein only (Lee et al., 1994). The NMR-determined solution-phase structures of various *P. aeruginosa* pilin peptides are also available (McInnes et al., 1993, 1994; Campbell et al., 1995). Our current study has investigated the contribution of specific residues for adhesin function.

Contribution of Individual Residues to the Adhesintope. Six side chains and the disulfide bridge (Cys^{129} and Cys^{142}) located in the C-terminal region of the PAK pilin were found to be critical for mediating binding to A549 cells and were considered to contribute to the adhesintope responsible for receptor interactions. These six side chains are Ser^{131}, Gln^{136}, Ile^{138}, Pro^{139}, Gly^{141}, and Lys^{144}, which are distributed throughout the C-terminal region of PAK pilin. Five of these residues are located inside the disulfide loop. This suggested that a conformational determinant is required for receptor interactions. Interestingly, of these six residues, three (Gln^{136}, Ile^{138}, Pro^{139}) are found in the epitope sequence (DEQFIPK) of monoclonal antibody PK99H, which specifically recognizes the pilin peptide $PAK(128-144)_{ox}$ (Wong et al., 1992). This observation suggests that PK99H blocks $PAK(128-144)_{ox}$ binding to the epithelial cells by binding to part of the adhesintope sequence recognized by the cell receptors. Because of the diversity of the residues found in the adhesintope, different types of interactions could be involved: for instance, hydrogen bonds (Ser^{131}, Gln^{136}), hydrophobic interactions (Ile^{138}, Pro^{139}), ionic interactions (Lys^{144}), and residues involved in conformational control (Pro^{139}, Gly^{141}, and the two cysteines involved in the disulfide bridge, Cys^{129} and Cys^{142}). In the case of the KB7 pilin peptide, nine residues were found to be important for the KB7 adhesintope; they are Ala^{130}, Thr^{131}, Thr^{132}, Val^{133}, Asp^{134}, Ala^{135}, Lys^{136}, Arg^{138}, and Pro^{139}. These residues also involve similar interactions such as hydrogen bonds (Thr^{131}, Thr^{132}), hydrophobic interactions (Ala^{130}, Val^{133}, Ala^{135}), and ionic interactions (Asp^{134}, Lys^{136}, Arg^{138}). The amino acid residues involved in A549 receptor binding are summarized in Table 3.

Effects of Hydrophobic Side Chains on Adhesin−Receptor Interactions. An unexpected observation obtained from the adhesintope mapping result is the nonessential phenylalanine residue at position 137. This hydrophobic residue is highly conserved among different *P. aeruginosa* strains (Paranchych et al., 1990) and was found to be very critical for antibody binding (Wong et al., 1992). However, our data from whole cell binding studies showed that Phe^{137} was not involved in pilus−cell receptor interactions as substitution of Phe^{137} by an alanine did not result in any decrease in the binding affinity (Table 1). Another hydrophobic residue found in the PAK sequence, Ile^{138}, contributed to the interactions between the pilin adhesintope and the cell-surface receptors. However, Ile^{138} is mutated to a positively charged residue, arginine, in the KB7 sequence. Thus, it becomes important to determine if Arg^{138} contributed to the adhesintope in KB7 or if the conserved Phe^{137} adjacent to Ile^{138} in the PAK sequence provided the hydrophobic interactions in the KB7 adhesintope, that is, whether a loss of the Ile side chain could be compensated for by a shift of the hydrophobic interaction to Phe^{137} in the KB7 peptide. From the adhesintope map (Table 1), it was striking that KB7(F137A) bound as well as the native KB7 peptide to A549 cells (I_{50} values of 140 and 190 μM, respectively). Moreover, the KB7 17-residue sequence contains another hydrophobic residue (valine) at position 133. Removal of this hydrophobic side chain by alanine replacement caused a significant decrease in binding affinity [I_{50} > 960 μM for KB7(V133A); Table 1] to A549 cells. It seems that Val^{133}, instead of Phe^{137}, may contribute to the hydrophobic interaction between the KB7 peptide and the cell receptor. In addition, the Arg^{138} in the KB7 peptide was found to be important for receptor binding since substitution of this residue by alanine greatly reduced binding to A549 cells [I_{50} of KB7(R138A) was >980 μM]. Further, the Arg^{138} was found to be unique to the KB7 sequence and substitution of Arg in the PAK sequence, PAK(I138R), decreased binding affinity to A549 cells (I_{50} > 720 μM; Table 2).

Structure−Function Relationship of the Adhesintope: cis/trans Conformation. Recently, the solution structure of the *trans* isomer of the oxidized PAK C-terminal 17-residue pilin peptide has been deduced by 2-D ^1H NMR spectroscopy (McInnes et al., 1993, 1994). The PAK peptide appears to have two isomers, the *cis* and *trans* configurations, due to the isomerization of the Ile^{138}−Pro^{139} amide bond (McInnes et al., 1994). In the oxidized form, the *trans* isomer is dominant over the *cis* by a ratio of 3:1 at 5 °C. In the reduced form, the *trans*:*cis* ratio is 14:1. Therefore, formation of the intramolecular disulfide bridge between Cys^{129} and Cys^{142} shifts the equilibrium in solution toward the *cis* isomer. It has been proposed that the *trans* conformation is more favorable for antibody interaction (McInnes et al., 1994) since the reduced PAK peptide and a truncated peptide without the two cysteine residues bind with equal affinity as the oxidized peptide to monoclonal antibody PK99H (Wong et al., 1992). In contrast, the *cis* isomer seems to be more important for receptor binding, as the reduced peptide has a lower binding affinity as compared with the oxidized peptide (Irvin et al., 1989). In Table 2, the PAK peptide analog with both cysteine residues substituted by alanine shows a large decrease in binding affinity to A549 cells. In addition, substitution of Pro^{139} by alanine in the PAK peptide has been shown to cause a disappearance of the *cis* configuration (McInnes et al., 1994), and this effect can be seen with PAK(P139A) where it loses its binding affinity to A549 cells (Table 1). These results agree with the hypothesis that the *cis* isoform of the PAK pilin peptide is important for receptor interaction.

Table 4: Summary of the Change in Chemical Shift and Hydrogen-Bonding Properties of the Amino Acid Residues in the *cis* and *trans* Isomers of PAK(128−144)$_{ox}$

				131					136	138	139		141			144
PAK sequence		K	C	T	S	D	Q	D	E	Q	F	I	P	K	G	C	S	K
Change in chemical shift ($\Delta\delta$)[a]		·	·	Δ	·	·	·	·	·	Δ	Δ	Δ	·	·	Δ	·	·	·
H-bond protection from exchange with solvent[b]:	*trans*	·	·	·	·	·	·	·	·	·	p	·	·	·	·	p	·	·
	cis	·	·	·	p	·	·	·	p	p	·	·	·	·	·	p	·	·
PAK adhesintope[c]		·	⊕	·	*	·	·	·	·	*	·	*	*	·	*	⊕	·	*

[a] Residues for which $|\Delta\delta| > 0.2$ ppm as compared between the *cis* and *trans* isomers of PAK(128−144)$_{ox}$ at 5.0 °C (McInnes et al., 1994). The significant residues are denoted by a Δ. [b] As deduced from the temperature coefficient ($-\Delta\delta/\Delta T$ ppb/K) measurements of the amide proton (McInnes et al., 1994). Where $-\Delta\delta/\Delta T < 5$ ppb/K indicates a protected amide (Dyson et al., 1985). Residues with possible hydrogen-bonding properties are denoted by a p. [c] Residues involved in A549 cell binding (Table 1) are denoted by an *. The cysteine residues involved in disulfide bond formation are critical for receptor binding (⊗).

From the NMR studies, five residues show a large change in the amide proton chemical shift between the *trans* and *cis* conformations; they are Thr^{130}, Gln^{136}, Phe^{137}, Ile^{138}, and Gly^{141} (Table 4). Three of them, Gln^{136}, Ile^{138}, and Gly^{141}, also constitute part of the PAK adhesintope sequence (Table 1). The large chemical shift difference observed for Thr^{130} between the *cis* and *trans* isomers is mainly due to the hydrogen bond contributed by Ser^{131} in the *cis* isomer (as deduced from the temperature coefficient; Table 4), which is not present in the *trans* isomer. Thr^{130} does not appear to be involved in binding since substitution of this residue by alanine does not change the binding affinity of the peptide (Table 1). Similarly, the change in chemical shift found in Gln^{136} is also due to the putative hydrogen bond that occurs in the *cis* conformation but not in the *trans*. Since the PAK peptide is assumed to bind as a *cis* isomer, one could suggest that the hydrogen-bonding properties of Ser^{131} and Gln^{136} are important in the binding process. Therefore, this may explain why these two residues are important to the adhesintope.

Interestingly, the *cis* configuration does not seem to be very significant for KB7 peptide binding. Only a very small proportion (<5%) was observed by NMR study (Campbell et al., 1995). It is plausible that the KB7 peptide utilizes the *trans* isoform for receptor interaction, whereas the PAK strain makes use of the *cis* isoform for receptor binding.

Structure−Function Relationship of the Adhesintope: Secondary Structure. As the solution structure of the *trans* isomer of PAK(128−144)$_{ox}$ peptide was generated from the NMR data, two different types of β-turns were observed (McInnes et al., 1993; Campbell et al., 1995). A type I β-turn was identified from residue 134 to residue 137 corresponding to the sequence DEQF, whereas a type II β-turn was found from residue 139 to residue 142 with the sequence PKGC. These two β-turns thus form a structurally more rigid region in the flexible peptide. Interestingly, the epitopic sequence, DEQFIPK, for the monoclonal antibody PK99H (Wong et al., 1992) and part of the PAK adhesintope are located in these rigid regions.

The C-terminal region of KB7 pilin is only semiconserved as compared to the PAK sequence, and along the 17-residue region, only six residues (including the two cysteines) are conserved. In spite of this, the secondary structure of the KB7 peptide is quite similar to that of PAK as revealed by NMR studies (Campbell et al., 1995). It was found that the KB7 peptide, like the PAK peptide, has two β-turn structures in sequences 134−137 (DAKF) and 139−142 (PNGC), respectively. However, when the backbone structures of these two peptides are aligned to each other, the side chain of Phe^{137} in the KB7 peptide faced to a different orientation. In addition, the phenylalanine side chain is also more buried in the KB7 structure (Campbell et al., 1995). The dissimilarity of the Phe^{137} in the solution structure of these two pilin strains is in close agreement with our result that Phe^{137} is not involved in binding in both peptides (Table 1).

The importance of the secondary structure involved in A549 cell binding was examined. In the case of the PAK pilin peptide, four out of the six amino acid residues found in the PAK adhesintope sequence are located in a structurally more rigid region of the flexible peptide. Two of these residues, Pro^{139} and Gly^{141}, are located at the i and $i+2$ positions of the type II β-turn. Gly^{141} at position $i+2$ is a very important residue in constructing the type II β-turn; substitution of this residue by alanine will disrupt the formation of a type II β-turn. Similarly, substitution of Pro^{139} by alanine at position i will also lower the chance of a type II β-turn formation (Wilmot & Thornton, 1988). Therefore, it is possible that the type II β-turn is an important structure motif required for receptor binding and that both Gly^{141} and Pro^{139} are important for the PAK pilin peptide binding to A549 cells because these two residues are required to maintain the secondary structure of a type II β-turn.

In contrast, the type I β-turn may not be as important as the type II β-turn for the PAK pilin peptide binding to A549 cells. There is only one PAK adhesintope residue found in the type I β-turn region. Gln^{136} was found at the $i+2$ position of the type I β-turn. However, the type I β-turn can tolerate a mutation to an alanine at this position according to the prediction parameters developed by Wilmot and Thornton (1988). It is plausible that the substitution of Gln^{136} disrupts the hydrogen-bonding property of this residue rather than directly affects the secondary structure of the peptide, since the amide proton of Gln^{136} showed a lowered temperature coefficient (McInnes et al., 1993).

However, the whole scenario is quite different in the case of the KB7 pilin peptide. Four adhesintope residues were found in the two β-turn regions. Asp^{134}, Ala^{135}, and Lys^{136} are located at the i, $i+1$ and $i+2$ positions of the type I β-turn, whereas Pro^{139} is found at the i position of the type II β-turn. Since substitution of Gly^{141} in the KB7 sequence is permissible for receptor binding, it seems that the type II β-turn is not as important for the KB7 peptide to interact with the A549 cell receptor. Indeed, more adhesintope residues were found at the N-terminal side of the KB7 peptide and are close to the type I β-turn. These residues are found on the same side of the KB7 peptide as determined from the NMR structure. Therefore, the type I β-turn is probably more important for the KB7 pilin peptide interaction with its receptor.

Compensatory Mutation of the KB7 Adhesintope Sequence. The interactions between the C-terminal peptide of the PAK pilin and the A549 cells require a conformational adhesintope, which contains the amino acid residues Ser^{131}, Gln^{136}, Ile^{138}, Pro^{139}, Gly^{141}, and Lys^{144} and the disulfide bridge

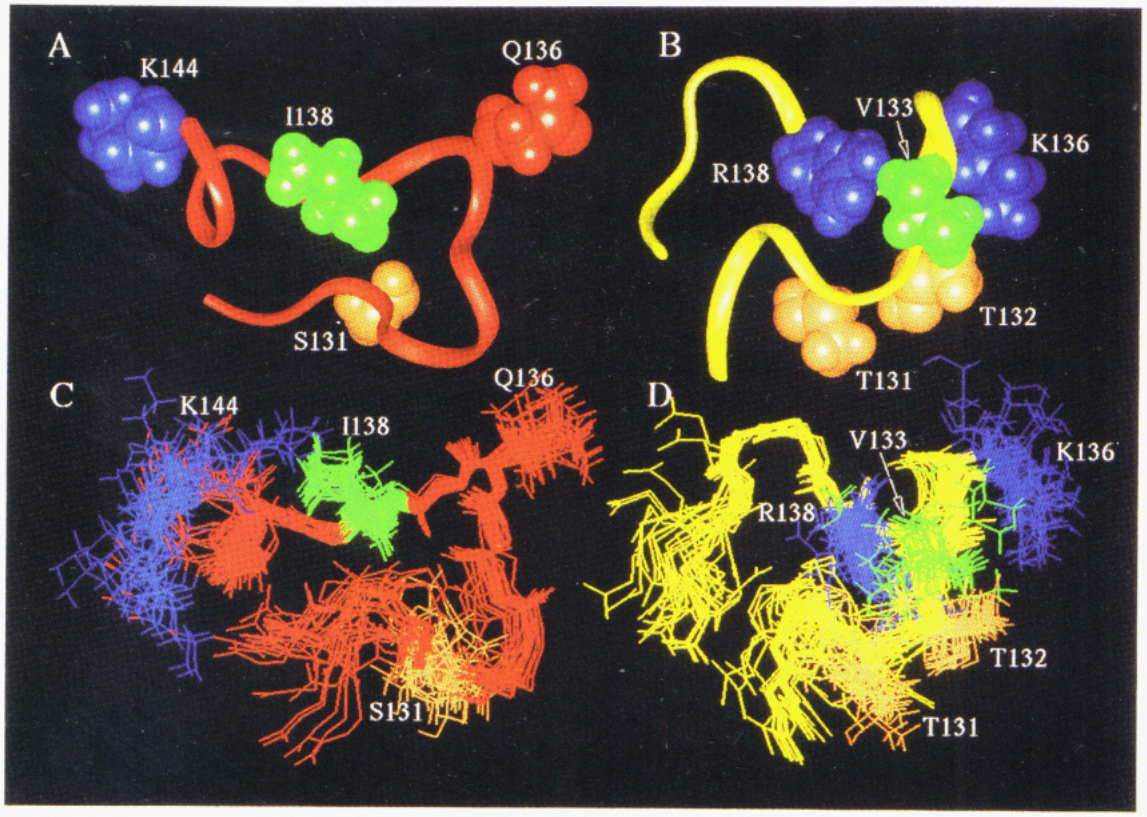

FIGURE 4: NMR solution structure of the PAK pilin peptide (panels A and C) and the KB7 pilin peptide (panels B and D) corresponding to the C-terminal sequence 128−144 of the pilin proteins. Panels A and B display the energy-minimized average structure of the PAK and KB7 peptides. The side chains of some of the adhesintope residues are color coded and shown in a space-filled presentation. The backbone of the two peptides is displayed as a solid ribbon (red for PAK and yellow for KB7). The two pilin peptides are aligned according to the orientation of the hydrophobic side chains (Ile^{138} and Val^{133}), the polar side chains (Ser^{131} and Thr^{131}), and the cationic side chains (Lys^{144} and Arg^{138}) of the PAK and KB7 peptides, respectively (see text). Panels C and D display the disposition of the adhesintope side chains from the ensemble of simulated annealing structures of the PAK and KB7 peptides with backbone atoms superimposed from residue 134 to residue 142.

(Tables 1 and 2). Two structural requirements, such as a *cis* conformation and the presence of type I and type II β-turns, are proposed. These structural requirements require the presence of an intrachain disulfide bridge between Cys^{129} and Cys^{142} and the residues Pro^{139} and Gly^{141}. Other residues, such as Ser^{131} and Gln^{136}, may contribute to hydrogen bond formation, whereas Ile^{138} may be involved in hydrophobic interaction and Lys^{144} may provide a positive charge for receptor interaction. Similarly, nine residues were important for the KB7 pilin peptide binding to A549 cells; these residues are Ala^{130}, Thr^{131}, Thr^{132}, Val^{133}, Asp^{134}, Ala^{135}, Lys^{136}, Arg^{138}, and Pro^{139}. When these residues are compared with those of the PAK adhesintope, it seems likely that "compensatory mutations" occurred to give rise to the KB7 sequence, in which different mutations work together to maintain the functionality of the adherence binding domain. Multiple mutations such as Thr^{130}→Ala, Ser^{131}→Thr, Asp^{132}→Thr, Gln^{133}→Val, Glu^{135}→Ala, Gln^{136}→Lys, and Ile^{138}→Arg are required in order for the KB7 pilin to maintain the side chain and structural requirements for receptor interactions, while a single mutation is not permissible as in the case of PAK(I138R) (Table 2). Interestingly, the major hydrophobic interaction with the receptor and the PAK adhesin involves Ile^{138}. In KB7, the only hydrophobic residue is Val^{133}. These results suggest the importance of this hydrophobic interaction with the receptor and that either a conformational change in the receptor is required to bind the different strains of this pathogen or a rotation of the receptor binding domain on the pathogen of different strains is required to present a similar surface to the receptor as shown in the model described below.

In order to investigate how the two heterogeneous but functionally similar pilin peptides interact with the receptor, the NMR solution structures of the peptides were determined and compared (Figure 4). The orientations of the major hydrophobic side chains of the PAK and KB7 peptides (Ile^{138} and Val^{133}, respectively) involved in receptor binding were employed as a reference point, upon which other side chains were aligned so as to obtain a maximum number of side chains with similar orientation. A cluster of amino acid side chains such as Lys^{144}, Ser^{131}, and Ile^{138} in the PAK peptide (Figure 4A) seems to form a similar binding surface as Arg^{138}, Thr^{131}, and Val^{133} in the KB7 sequence (Figure 4B). Flexibility of the side chains was also determined by displaying the ensemble of simulated annealing structures of both peptides (Figure 4C,D). The side chain of Lys^{144} in the PAK peptide is very flexible (Figure 4C), which is important for the peptide to form a similar binding surface as that of the KB7 peptide. However, other side chains such as Gln^{136} in PAK and Thr^{132}, Asp^{134}, and Lys^{136} in KB7 are oriented quite differently. This has led to the postulation that the pilin receptor may contain more sites than each peptide can occupy. In other words, receptor binding occurs when each pilin peptide interacts with a particular combination of binding sites in the receptor. A schematic model is shown in Figure 5. For example, if the model receptor contains seven different side-chain binding sites, then, during receptor interaction, the PAK peptide occupies four of the

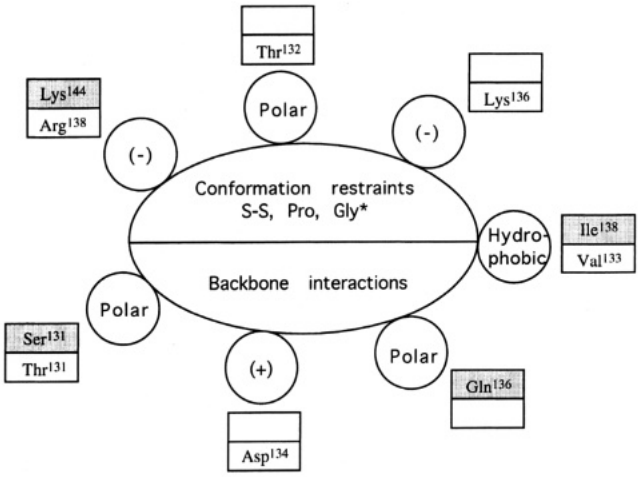

FIGURE 5: Hypothetical model showing the proposed compensatory mutations adapted by the PAK and KB7 pilin peptides in receptor binding. The model receptor is proposed to contain different kinds of binding sites such as the cationic (+), anionic (−), polar, and hydrophobic sites, which are denoted by small circles. In addition, other binding requirements such as backbone interactions and conformational restraints of the peptides are also proposed and indicated by an oval. The conformational restraints are conferred by the intrachain disulfide bridge of the peptide (S−S), Pro^{139}, and Gly^{141} (*Gly^{141} is only important for the PAK peptide). The amino acid side chains of both peptides involved in receptor interactions are boxed, whereas the PAK side chains are shaded and the KB7 side chains are left unshaded. An empty box denotes unavailability of the side chain for receptor interaction at that particular site.

binding sites, whereas the KB7 peptide occupies six. Among them, three binding sites are common to both peptides (Figure 5). Deletion of any of the adhesintope side chains by alanine substitution will greatly affect the binding efficacy. When mutations in the pilin protein occur that decrease binding, compensatory mutations must also be present to fill other available binding sites in the receptor to maintain binding affinity. In addition, other requirements such as conformational restraints of the peptide and backbone interactions are equally important. Conformational restraints can maintain the secondary structures such as the two β-turns found in all pilin peptides studied to date, which are important for the presentation of the side chains and the peptide backbone for receptor binding. Loss of binding has been observed when the intrachain disulfide bridge of the pilin peptide was removed or when the conformational control residues such as Pro^{139} (for both peptides) and Gly^{141} (for PAK only) were substituted by an alanine. Though the present working model explains the data, conformational change of the receptor cannot be ruled out in the interaction of different pilin strains with the receptor.

It is of interest to note that sequence homology is not necessarily a prediction of the adhesintope. Aside from the Pro^{139} and Gly^{141} residues, Phe^{137} is also a conserved residue found in the *P. aeruginosa* pilin sequences among different strains (Paranchych et al., 1990); however, Phe^{137} is not involved in receptor interaction. Furthermore, the epitope deduced for a specific anti-pilus antibody is not a mimic for the adhesintope. When the PK99H epitope is compared with the PAK adhesintope, there are only two common residues (Ile^{138} and Pro^{139}) critical to binding in both cases.

In conclusion, the amino acid residues required for both PAK and KB7 pilin binding to the A549 cell were elucidated. This information sheds light on how the pilus adhesin interacts with the epithelial cell receptors and enables us to understand the structure−function relationship between *P. aeruginosa* pilus binding to its receptors and the ability of antibodies to block adherence. This knowledge will be important for designing therapeutics and cross-protective vaccines that prevent *P. aeruginosa* infections.

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