Site-Specific Conjugation to Interleukin 4 Containing Mutated Cysteine Residues Produces Interleukin 4–Toxin Conjugates with Improved Binding and Activity

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ABSTRACT: Fusion of a ligand to another protein frequently impairs the binding of the ligand. Recombinant toxins composed of mutants of *Pseudomonas* exotoxin (PE) fused to the C-terminus of human interleukin 4 (IL4) are cytotoxic to IL4 receptor- (IL4R-) bearing tumor cells but bind to the IL4R with only 1% the affinity of IL4. We have developed a method to connect a toxin to a ligand which allows the junction to be moved to a location on the ligand which would minimize the binding impairment. We designed mutants of IL4 in which residue 28, 38, 68, 70, 97, or 105 was substituted with cysteine. All purified mutants bound to the IL4R with 60–100% the affinity of IL4, indicating that the IL4 structure was essentially unchanged. The IL4 mutants were then each conjugated through a disulfide bond to PE35, a truncated form of PE which contains a single cysteine. IL4 conjugated to PE35 at residue 28, 38, or 105 of IL4 bound with 10-fold improved affinity and was 10-fold more cytotoxic than the recombinant IL4–toxin in which PE is fused to position 129 at the C-terminus of IL4. IL4 containing PE35 conjugated at position 68, 70, or 97 had lower binding affinity and cytotoxic activity. These results indicate that the location of the ligand–protein junction can be selectively moved to enhance conjugate effectiveness, and implications could be made regarding which regions of IL4 are important for binding.

Fusing two proteins together to make a multifunctional molecule commonly impairs the function of at least one of the two components. For example, fusion of molecules such as interleukin 2, interleukin 3, interleukin 6, transforming growth factor α, and granulocyte–macrophage colony stimulating factor to other proteins has been reported to decrease binding affinity by 20–100-fold (Curtis et al., 1991; Edwards et al., 1989; Jean et al., 1993; Williams et al., 1990). Whether the reduction in ligand binding affinity is due to impaired protein folding or blocking of binding to a receptor, the location of the ligand–protein junction could be an important factor. We have studied this important problem using fusions of interleukin 4 with derivatives of *Pseudomonas* exotoxin.

Interleukin 4 (IL4) is an important growth factor for both T and B cells (Yokota et al., 1986; Howard et al., 1982). IL4 receptors (IL4R's) are overexpressed on many B- and T-cell lymphomas (Park et al., 1987; Mori et al., 1993) and on activated T lymphocytes which mediate autoimmune diseases (Mozo et al., 1993). IL4R's have also been found on many solid tumors, such as gastric cancer (Morisaki et al., 1992), malignant melanoma, ovarian and breast carcinoma, malignant mesothelioma (Obiri et al., 1994), and renal cell carcinomas (Obiri et al., 1993; Varricchio et al., 1993). The X-ray crystallographic and NMR structures of human interleukin 4 (IL4) have been solved (Walter et al., 1992; Powers et al., 1992, 1993) and show that the 129 amino acid protein is composed mainly of four α helices. The C-terminal region of IL4 is apparently important for binding, since a peptide corresponding to this region elicited IL4 antibodies which blocked receptor binding (Le et al., 1991). By IL4's sequence homology to murine IL4 and from mutagenesis studies of murine IL4, residues L^{109}, N^{111}, F^{112}, and L^{116} of human IL4 were suggested as important for receptor binding (Morrison & Leder, 1992). Immunochemical mapping and mutagenesis have recently suggested that residues 104–129, 70–92, and 61–82 and in particular residues K^{84}, R^{88}, and N^{89} are important for receptor binding, while residues 43–59 are not (Ramanathan et al., 1993). Finally, residues important for binding have been derived from IL4–IL4R models based on either the structural homology of IL4 and hGH or the assumed structural homology of IL4R and CD4 (Powers et al., 1993; Bamborough et al., 1993).

Pseudomonas exotoxin (PE) is a 613 amino acid protein toxin which binds to cells, internalizes, translocates to the cytosol, and kills the cell by inhibition of protein synthesis. The domains of PE which perform these functions have been defined by X-ray crystallography (Allured et al., 1986) and by mutagenesis studies (Hwang et al., 1987). Domain Ia (amino acids 1–252) binds to the PE receptor, identified as the α2 macroglobulin receptor (Kounnas et al., 1992). After internalization, domain II (amino acids 253–364) undergoes proteolytic cleavage between residues 279 and 280 (Ogata et al., 1992) and translocates the C-terminal fragment (amino acids 280–613) to the cytosol (Ogata et al., 1990). Domain III (amino acids 400–613) contains the enzyme which ADP-ribosylates elongation factor 2.

To target particular cells, we have connected ligands to mutated forms of PE which cannot bind to the PE receptor (Pastan et al., 1992). PE^{4E} is a full-length mutated form of PE which contains four glutamate mutations in domain Ia (Chaudhary et al., 1990b). PE40 is a truncated form of PE which is missing domain Ia. PE38, a truncated form of PE40, is missing both domain Ia and amino acids 365–380 from domain Ib. This region contains a disulfide bond which is unnecessary for cytotoxicity (Siegall et al., 1989; Kreitman et al., 1993). PE35 is an even smaller version that contains

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Table 1: Oligonucleotides Used in Plasmid Construction[a]

BBK9	3′-tgc-aag-cca-aga-ccg-tgg-ttc-gac-ctc-gag-ttt-cca-cca-ccg-ccg-tcg-gtt-cga-agc-gac-tgg-cgc-gtg-gtc-5′
BK10	5′-c-aaa-gga-ggc-ggt-ggc-tcg-ggc-ggt-ggc-ggc-tcg-ggt-ggc-ggc-ggc-tct-ca-3′
BK11	3′-tc-gag-ttt-cct-ccg-cca-ccg-agc-ccg-cca-ccg-ccg-agc-cca-ccg-ccg-ccg-aga-gtt-cga-5′
BK12	5′-act-ata-ggg-aga-cca-caa-cgg-ttt-3′
BK52	5′-ccc-act-tgc-ctt-taa-gaa-gga-gat-ata-3′
BK64	3′-cgt-tat-tga-tcg-tat-tgg-gga-5′
BK78	5′-ata-cga-ctc-act-ata-ggg-aga-3′
BK81	3′-ttt-ata-agt-ttc-aca-agc-tcg-ctc-gag-ctt-cga-aag-5′
BK87	5′-cgg-cca-cga-tgc-gtc-cgg-cgt-3′
BK90	3′-gcg-acg-gac-cca-cgc-tgt-aca-gtc-gtc-aag-gtg-tcc-5′
BK95	3′-ttt-ata-agt-ttc-aca-agc-tcg-att-cga-att-cga-aag-5′
BK98	3′-ttc-ctg-tga-gcg-acg-gag-ccc-aca-tga-cgt-gtc-gtc-aag-5′
BK99	3′-tag-aaa-cga-cgg-agg-ttc-aca-tgt-tga-ctc-5′
BK100	3′-acc-ccg-gac-cgc-ccg-aac-aca-agg-aca-gga-5′
BK102	3′-tga-gac-acg-tgg-ctc-aac-acg-cat-tgt-ctg-5′
BK103	3′-agg-aca-gga-cac-ttc-ctt-cgt-acg-gtc-tca-tgc-5′

[a] The cloning sites for *Hin*dIII and *Sac*I are underlined.

only the translocated form of PE38 (Met and amino acids 281–364 and 381–613) and has only one cysteine residue at position 287. For optimal cytotoxic activity, ligands are fused to the amino terminus of PE^{4E}, PE40, or PE38. PE35 must have a free N- and C-terminus to be cytotoxic and has been conjugated to antibodies via a disulfide bond to form active immunotoxins (Theuer et al., 1993).

IL4 has recently been fused to the N-terminus of PE^{4E} and the resulting chimeric toxin, IL4–PE^{4E}, was shown to be cytotoxic toward T- and B-cell lines and activated T-lymphocytes (Debinski et al., 1993). IL4 has also been fused to PE38QQR, which contains amino acids 253–364 and 381–613 of PE with mutations of lysines at residues 590, 606, and 613 to Q, Q, and R, respectively (Debinski & Pastan, 1992). IL4–PE38QQR was also cytotoxic toward target cells and was much less toxic to mice than IL4–PE^{4E} (Debinski et al., 1994). Although quite cytotoxic, we have found that IL4–PE^{4E} and IL4–PE38QQR bind to the IL4R with greatly diminished affinity, suggesting that the bulky toxin blocked receptor binding when fused to the IL4 C-terminus (Debinski et al., 1993).

In the present study, we developed a site-specific conjugation procedure to design new IL4–toxins with improved binding and cytotoxic activity. To accomplish this required several steps including (1) introducing a seventh cysteine in one of several locations within IL4 and determining how well each IL4-Cys mutant bound to the IL4R, (2) site-specific conjugation of PE35 to each of the IL4-Cys mutants through a disulfide bond, and (3) testing purified IL4–PE35 mutants for binding and cytotoxic activities to determine which region of IL4 can be connected to a toxin with the least disruption of receptor binding.

MATERIALS AND METHODS

Plasmid Construction. In vitro mutagenesis using the polymerase chain reaction (PCR) was performed with the Gene-Amp kit (U.S. Biochemical Corp., Cleveland, OH). PCR reactions included denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and polymerization at 72 °C for 3 min. Thirty cycles were used, with a 10-s extension of polymerization per cycle. Synthetic oligonucleotides used are shown in Table 1. The DNA sequence of the T7 promoter, Shine–Delgarno sequence, and human IL4 template is shown in Figure 1. Plasmid sequencing was performed using the Sequenase 2.0 kit (U.S. Biochemical Corp.) or a Dyedeoxy terminator cycle sequencing kit and an automated sequencer (Applied Biosystems, Foster City, CA).

Recombinant single-chain IL4–toxins described previously include IL4–PE^{4E} and IL4–PE38QQR, encoded by pWDMH4 and pWDMH4.38Q, respectively (Debinsky et al., 1993; 1994). In this study, two new single-chain IL4–toxins, encoded by pRKL418 and pRKL428, were constructed containing the connectors ELK(G_4S)QAF and ELKAF(G_4S)$_3$QAF, respectively, between the C-terminus of IL4 and PE40. These connectors were originally cloned between anti-Tac(Fv) and PE40. p7018, encoding anti-Tac(Fv)–PE40 (Batra et al., 1990) was amplified using primers BK12 and BK9. This amplified fragment extended from the 12th base of the T7 promoter to the last amino acid of anti-Tac(Fv) (Batra et al., 1990) and was followed by the codons ggt-ggt-ggc-ggc-agc-caa-gct-tcg-ctg-acc-gcg-cac-cag, which encode the linker (G_4S) and contain a *Hin*dIII site. The amplified fragment was cut with *Xba*I and *Hin*dIII and the 0.77-kb fragment was ligated to the 4.2-kb *Hin*dIII–*Xba*I fragment of p7018. The resulting plasmid, pRK718, encodes anti-Tac(Fv)–(G_4S)QAF–PE40. To make pRK728, encoding anti-Tac(Fv)–AF(G_4S)$_3$-QAF–PE40, oligonucleotides BK10 and BK11 were annealed and the duplex was ligated to *Hin*dIII-digested p7018. All plasmids encoding anti-Tac(Fv) contain a *Sac*I site within the sequence gag-ctc-aaa which encodes the last three amino acids of anti-Tac(Fv).

pWDMH4 was amplified with BK78 and BK81, producing a fragment containing the T7 promoter and IL4 codons, followed by the bases gag-ctc-gaa-gct-ttc, which contain a *Sac*I site. The 0.44-kb *Xba*I–*Sac*I fragment of this amplified sequence was ligated to the 4.2-kb *Sac*I–*Xba*I fragment of pRK718, the 4.2-kb *Sac*I–*Xba*I fragment of pRK728, or the 3.0-kb *Sac*I–*Xba*I fragment of pVCDT1-anti-Tac(Fv) [encoding DT388-anti-Tac(Fv)] (Chaudhary et al., 1990a), resulting in pRKL418, pRKL428, or pRKL4EL, respectively. The latter plasmid encodes IL4 followed by the amino acids ELKA.

To make pRKL4, encoding native IL4, pRKL4EL was amplified with primers BK78 and BK95 (Figure 1, Table 1). The 0.43-kb *Xba*I–*Hin*dIII fragment of the amplified sequence was ligated to the 3.0-kb *Hin*dIII–*Xba*I fragment of pVCDT1-anti-Tac(Fv).

To construct plasmids encoding mutants of IL4 which each contain an extra cysteine, we used a recently described method of site-directed PCR mutagenesis by overlapping amplified fragments which require only one new primer for each mutant (Mikaelian & Sergeant, 1992). The template for each mutant was pRKL4EL and Figure 1 shows its sequence in addition to where the relevant primers anneal, and Figure 2 shows a scheme for PCR and plasmid construction. In the first step, pRKL4EL was amplified by BK87 (or BK78 in the case of pRKL4C70) and the mutagenic primer. The mutagenic primer was BK102, BK99, BK98, BK90, BK100, or BK103 to produce an amplified fragment encoding a new cysteine at position 28, 38, 68, 70, 97, or 105, respectively. In a second step, pRKL4EL was amplified by BK52 and BK64. As shown in Figure 2, the 5′ end of the amplified fragment in the second step was made not to anneal to any of the amplified fragments in the first step. The primers of each amplified fragment were removed by passage through a single-strand DNA-binding column (Advanced Genetic Technologies Corp., Gaithersburg, MD), concentration on Centricon-100 (Amicon, Beverly, MA), and purification on low melting point SeaPlaque GTG agarose (FMC, Rockland, ME). Approximately 1:1

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-57          BK87->
CGG TGA TGC CGG CCA CGA TGC GTC CGG CGT AGA GGA TCT TGA GAT CTC GAT CCG CGA AAT

BK78->                                    33       XbaI            BK52->
ATA CGA CTC ACT ATA GGG AGA CCA CAA CGG TTT CCC TCT AGA AAT AAT TTT GTT TAA CTT

63                                        93/4
TAA GAA GGA GAT ATA CAT atg cac aag tgc gat atc acc tta cag gag atc atc aaa act
                     met his lys cys asp ile thr leu gln glu ile ile lys thr

123/14                                    153/24                  <-BK102
ttg aac agc ctc aca gag cag aag act ctg tgc acc gag ttg acc gta aca gac atc ttt
leu asn ser leu thr glu gln lys thr leu cys thr glu leu thr val thr asp ile phe

183/34                  <-BK99            213/44
gct gcc tcc aag aac aca act gag aag gaa acc ttc tgc agg gct gcg act gtg ctc cgg
ala ala ser lys asn thr thr glu lys glu thr phe cys arg ala ala thr val leu arg

243/54                                    273/64                          <-BK98
cag ttc tac agc cac cat gag aag gac act cgc tgc ctg ggt gcg act gca cag cag ttc
gln phe tyr ser his his glu lys asp thr arg cys leu gly ala thr ala gln gln phe

 <-BK90                                   333/84
cac agg cac aag cag ctg atc cga ttc ctg aaa cgg ctc gac agg aac ctc tgg ggc ctg
his arg his lys gln leu ile arg phe leu lys arg leu asp arg asn leu trp gly leu

363/94              <-BK100               393/104     <-BK103
gcg ggc ttg aat tcc tgt cct gtg aag gaa gcc aac cag agt acg ttg gaa aac ttc ttg
ala gly leu asn ser cys pro val lys glu ala asn gln ser thr leu glu asn phe leu

423/114                                   453/124                 SacI
gaa agg cta aag acg atc atg aga gag aaa tat tca aag tgt tcg agc gag ctc aaa gct
glu arg leu lys thr ile met arg glu lys tyr ser lys cys ser ser glu leu lys ala

<-BK95                                    513
TGA ATT CGG CTG CTA ACA AAG CCC GAA AGG AAG CTG AGT TGG CTG CTG CCA CCG CTG AGC

543/181             <-BK64                573
AAT AAC TAG CAT AAC CCC TTG GGC CTC TAA ACG GGT CTT GAG GGG TTT TTT GCT GAA
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FIGURE 1: Nucleotide sequence of template pRKL4EL showing annealing positions for PCR primers. Primer sequences are shown in Table 1. The numbering of the bases assumes that base 1 is the beginning of the T7 promoter. Underlined segments represent the positions at which the indicated primers anneal to the template. The annealing positions of primers BK90 and BK98 and of BK100 and BK103 overlap. The 5′ end (9 bases) of primer BK52 and the 5′ end (15 bases) of primer BK95 do not anneal to the template.

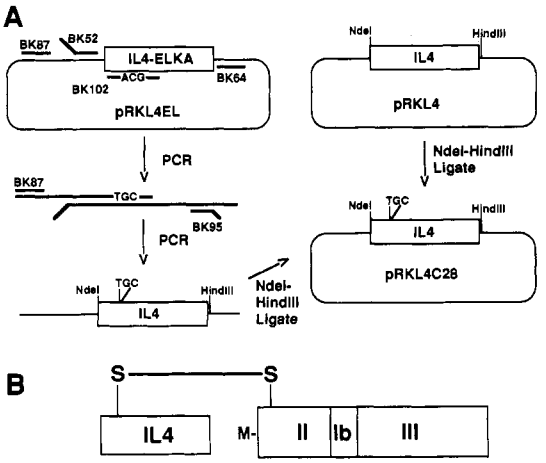

FIGURE 2: Scheme for construction of plasmids encoding IL4-Cys. (A) The template pRKL4EL encodes the mature 129 amino acid human IL4 protein followed by the amino acids ELKA (Figure 1). PCR is performed using primers BK87 and BK102, and then primers BK52 and BK64. Only the former reaction will yield double-stranded DNA encoding the new cysteine residue, but after PCR of both products using primers BK87 and BK95, only DNA encoding the desired mutation is obtained. Due to the sequence of BK95, this amplified DNA encodes the correct IL4 C-terminus and contains a *Hin*dIII site just after the stop codon. After digestion with *Nde*I and *Hin*dIII, the fragment is inserted into the vector derived from pRKL4, which encodes IL4. (B) The purified IL4-Cys mutant is conjugated to PE35, which contains methionine followed by amino acids 281–364 and 381–613 of PE. PE35 contains the C-terminal portions of domains II and Ib and all of domain III of PE.

mixtures of amplified fragment from the second step with one of the six amplified fragments from the first step were prepared by combining equal volumes of the fragments in low melting point agarose. A 1-μL aliquot of each mixture was used as a template in a third PCR reaction, using primers BK87 (or BK78 in the case of pRKL4C70) and BK95. The 0.43-kb *Xba*I–*Hin*dIII fragment of each of the six amplified sequences from the third PCR step was ligated to the 3.0-kb *Hin*dIII–*Xba*I fragment of pRKL4 [or the 3.0-kb *Hin*dIII–*Xba*I fragment of pVCDT1-anti-Tac(Fv) in the case of pRKL4C70]. The resulting plasmids, pRKL4C28, pRKL4C38, pRKL4C68, pRKL4C70, pRKL4C97, or pRKL4C105, encode IL4 containing an extra cysteine residue at position 28, 38, 68, 70, 97, or 105 of IL4, respectively.

Plasmid Expression and Protein Purification. Plasmids pWDMH4-38Q, pRKL418, and pRKL428 were expressed and protein was purified as described for IL4–PE^{4E} (Debinski et al., 1993). These proteins were all acidic and were purified by anion exchange followed by sizing chromatography.

Plasmids pRKL4, pRKL4EL, pRKL4C28, pRKL4C38, pRKL4C68, pRKL4C70, pRKL4C97, and pRKL4C105 encoding IL4 and IL4-Cys mutants were transformed into *Escherichia coli* BL21(λDE3) and grown in superbroth containing ampicillin (100 μg/mL), extra glucose (5 mg/mL), and extra $MgSO_4$ (1.6 mM). At an OD_{650} of ~3, isopropyl β-D-thiogalactopyranoside (IPTG, U.S. Biochemical Corp.) was added to a final concentration of 1 mM, and protein expression was induced for 90–120 min. The harvested cell paste from 1 L of culture was resuspended in 360 mL of TES (50 mM Tris-HCl pH 8.0, 20 mM EDTA, and 0.1 M NaCl) and lysozyme (13 mL of 5 mg/mL). After incubation for 30 min at 22 °C, DNase (0.8 mg in 0.8 mL of H_2O) was added, and after shaking and allowing to stand at 22 °C for 30 min,

the mixture was resuspended and centrifuged. The pellet was resuspended in 360 mL of TES + 40 mL of 25% Triton X-100 and the solution was incubated at 22 °C for 30 min and centrifuged. The pellet was washed two or three more times by resuspension in 360 mL of TES + 40 mL of 25% Triton X-100 and centrifugation and then washed four times by resuspension in 360 mL of TES and centrifugation. All resuspensions were performed using a Tissuemizer (Thomas Scientific, Swedesboro, NJ) at 22 °C, and centrifugations were performed at 4 °C in a GSA rotor (Sorvall, Wilmington, DE) at 13 000 rpm for 30–50 min. The purified inclusion body protein (~120 mg) was resuspended by sonication in 12 mL of 7 M guanidine hydrochloride containing 0.1 M Tris, pH 8, 2 mM EDTA, and 65 mM dithioerythritol (DTE, Sigma, St. Louis, MO) and incubated 8–24 h at 22 °C. The denatured reduced protein was clarified by centrifugation and diluted 100-fold into cold refolding buffer containing 0.1 M Tris, pH 8.0, 0.5 M L-arginine, 2 mM EDTA, and 0.9 mM oxidized glutathione (GSSG, Sigma, St. Louis, MO). After incubation at 10 °C for 36–48 h, the clear solution was dialyzed against 0.02 M NaOAc (pH ~4.8) to a conductivity of <5 mMho. The refolded dialyzed protein was clarified by filtration through a 0.45-μm filter and loaded onto ~10 mL of CM fast-flow cation-exchange resin (Pharmacia, Piscataway, NJ). The column was washed with equilibration buffer (0.02 M NaOAc, pH 4.8) and eluted with equilibration buffer containing 0.5 M NaCl. The IL4 mutants were >80% pure at this point (Figure 4A) but could be purified further by elution from a Mono S column (Pharmacia) using a linear NaCl gradient in equilibration buffer and/or by sizing chromatography. As expected, IL4-Cys mutants often appeared as a doublet on nonreducing SDS–PAGE (Figure 4A), attributed to the persistence of disulfide bonding to glutathionine (GSH) during refolding. This was confirmed by lack of reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent, Pierce, Rockford, IL). Protein concentrations were determined using the Pierce Coomassie Plus reagent (Rockford, IL). Bovine serum albumin was used as a standard for IL4–toxins, while IL4 was used as a standard for IL4-Cys mutants. IL4 was also quantitated using the previously published extinction coefficient 1.0 OD_{280} = 1 mg/mL (Kruse et al., 1992).

Chemical Conjugation. Disulfide chemical conjugates were prepared by three different techniques. In one method, PE35 in 0.1 M sodium phosphate buffer, pH 7, was derivatized with DTNB, forming a disulfide bond between the free sulfhydryl of PE35 and 2-nitro-5-thiobenzoic acid. IL4–PE35 could then be obtained by disulfide exchange after reaction with the IL4-Cys mutant. In the second method, the IL4-Cys mutant was reacted with 2 mM 2-mercaptoethanol at pH 7 for 45 min, which resulted in ~75% of the molecules having a free sulfhydryl, derivatized with DTNB, and reacted with reduced PE35 in 0.1 M sodium phosphate buffer, pH 7. In the third method, PE35 and the IL4-Cys mutant were simply mixed in 0.1 M sodium phosphate buffer, pH 8.0. In all conjugations IL4-Cys and PE35 were allowed to react overnight at 22 °C. In several conjugates, two or three different methods were used to prepare the same toxin, with no significant difference in cytotoxic activity (data not shown). In each preconjugation step, excess reducing agent or DTNB reagent was removed by passage of protein through PD-10 columns (Pharmacia). All conjugates were purified by Mono Q chromatography (Pharmacia).

Cytotoxicity Assays. Daudi and Raji-Tat (Puri & Aggarwal, 1992) cells were maintained in RPMI containing 10–15% fetal bovine serum (FBS). Cells suspended in RPMI + 10% FBS were plated in 0.05-mL aliquots of 4×10^4 cells in 96-well plates, and 0.05-mL aliquots of toxins or control molecules were added. After incubation for 20–24 h, the cells were pulsed with [^{3}H]leucine (1 μCi/well). After incubation for 4–6 h further, the cells were harvested onto protein-binding glass fibers filters (Wallac, Turku, Finland) and counted in a Beta-plate scintillation counter (Pharmacia-LKB, Gaithersburg, MD).

Binding Assays. For competitive binding assays, H9 human CD4+ T cells (2.5×10^6) were incubated in 126-μL aliquots of binding buffer (RPMI containing 10% FBS) with approximately 100 pM ^{125}I-IL4 and increasing concentrations of IL4, IL4-Cys mutants, or IL4–toxins for 1–2 h at 4 °C. The cell-bound radioactivity was isolated by centrifugation through a cushion of phthalate oils. The radioactivity from the tips of the polypropylene tubes was counted in a γ counter. The binding of IL4–toxins was also determined by a different binding assay. Daudi cells (1×10^6) were incubated in 200-μL aliquots of binding buffer with 100 pM ^{125}I-IL4 and increasing concentrations of IL4–toxin at 4 °C for 1–2 h. The cells were then centrifuged and washed with medium, and the recentrifuged cells were counted in a γ counter. Nonspecific binding, determined by incubating cells with >100-fold excess of unlabeled IL4, was subtracted from each value prior to determination of the concentration required for 50% displacement. To verify that the unlabled recombinant IL4 had full activity, its binding activity and appearance on SDS–PAGE was compared to that of clinical-grade IL4 (5×10^7 units/mg), kindly supplied by Immunex.

Computer Analyses. The nuclear magnetic resonance (NMR) structure of IL4 (Powers et al., 1992, 1993) was obtained from Dr. Marius Clore. The α-carbon backbone structure of hGH–hGHR (De Vos et al., 1992) was obtained from Dr. Anthony Kossiakoff. Structures were analyzed using an in-house molecular graphics program, GEMM.

RESULTS

We previously observed that recombinant fusions of mutant PE to the C-terminus of IL4 bind with greatly reduced affinity compared to IL4 (Debinski et al., 1993). To make an IL4–toxin with improved binding and hence improved cytotoxicity, two separate strategies were attempted. The first was to chemically conjugate truncated PE to IL4 mutants which contained an extra (seventh) cysteine at different locations. The second strategy was genetically inserting longer linkers between the IL4 C-terminus and truncated PE.

Construction of IL4 Mutants for Site-Specific Conjugation. To conjugate PE35 to specific residues in Il4, we mutated one residue in each new IL4 molecule to cysteine. The sites mutated, shown in Figure 3, were placed so as not to disturb the disulfide bonding structure of the molecule. The residues 28, 38, 68, 70, 97, and 105 are in connecting regions between the helices and were chosen mainly because of their distance from the C-terminus. Residues 38 and 105 are also potential glycosylation sites. Using a site-directed PCR–mutagenesis protocol (Mikaelian & Sergeant, 1992), plasmids were constructed which encoded a seventh cysteine at position 28, 38, 68, 70, 97, or 105. With this approach it was necessary to sequence the entire coding region of each of these plasmids, since cloning artifacts were frequently obtained in sites unrelated to where primers annealed (data not shown). The correct plasmids were expressed in *E. coli* and were refolded in a redox buffer. The dialyzed protein could be purified to >80–90% homogeneity by one-step elution from a cation-exchange resin.

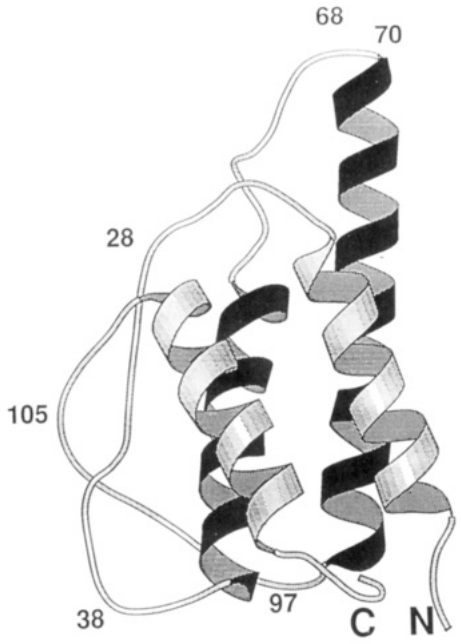

FIGURE 3: Three-dimensional structure of human IL4. The structure was provided by M. Clore from the NMR solution structure (Powers et al., 1992). The numbering system corresponds to the 129 amino acid mature recombinant protein, which has been crystallized (Walter et al., 1992). The ribbon diagram was generated with the program Molscript.

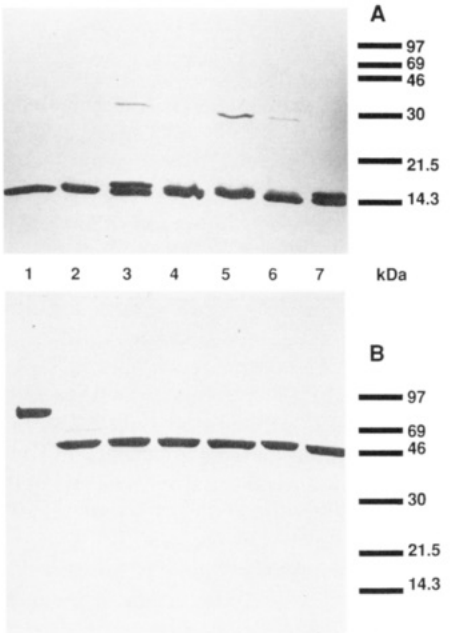

FIGURE 4: SDS–PAGE (nonreducing) of IL4 mutants (A, 15%) and IL4 toxins (B, 12%). In panel A, 2 μg was loaded of IL4 (lane 1), IL4C28 (lane 2), IL4C38 (lane 3), IL4C68 (lane 4), IL4C70 (lane 5), IL4C97 (lane 6), and IL4C105 (lane 7). The IL4 cysteine mutants were examined with DTNB and were found to consist of mixtures of molecules containing a free sulfhydryl and molecules where the free crysteine was connected through a disulfide bond to a small peptide, probably glutathione (0.3 kDa), which was used in the refolding solution. The percentage of molecules in each preparation which contained a free cysteine varied from 5% to 60%. In panel B, 3 μg was loaded of IL4–PE^{4E} (lane 1), IL4C28–PE35 (lane 2), IL4C38–PE35 (lane 3), IL4C68–PE35 (lane 4), IL4C70–PE35 (lane 5), IL4C97–PE35 (lane 6), and IL4C105–PE35 (lane 7).

Figure 4A depicts purified IL4 and the six IL4-Cys mutants renatured and purified by the same technique. The yields of purified monomer were approximately 30% of total recombinant protein or ~40 mg/L of culture. To achieve this yield, which is higher than the 1–2 mg/L of culture published previously (Kruse et al., 1991; Weigel et al., 1989), denatured protein is exposed to a redox buffer, where DTE and GSSG allow intermediate mixed-disulfide bonds to form between cysteine residues on the proteins and glutathione (Buchner & Rudolph, 1991). Several of the IL4-Cys mutants appeared as dimers on the nonreducing gel (Figure 4A). The slightly higher molecular weight band for each is attributed to IL4-Cys disulfide-bonded to glutathione, which adds an additional 0.3 kDa. Such mixed disulfides were previously detected when recombinant molecules containing free sulfhydryls were refolded in redox buffer containing glutathione (data not shown). The presence of a doublet on SDS–PAGE was associated with lack of reactivity of DTNB with a significant proportion of the purified molecules (data not shown).

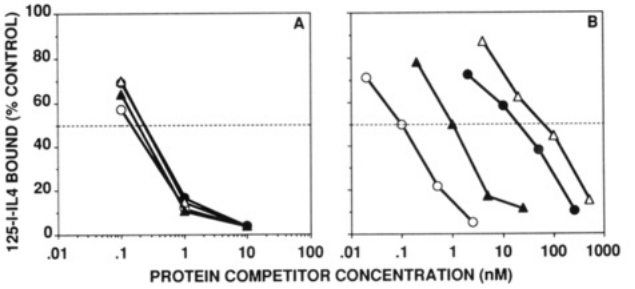

FIGURE 5: Binding of IL4 mutants and toxins to IL4R. In panel A, [^{125}I]-IL4 is displaced from H9 cells by the indicated concentrations of IL4 (○), IL4C70 (●), IL4C97 (△), and IL4C105 (▲). In panel B, 0.1 nM ^{125}I-IL4 is displaced from DAUDI cells by the indicated concentrations of IL4 (○), IL4C70–PE35 (●), IL4C97–PE35 (△), and IL4C105–PE35 (▲). The dotted line indicates 50% displacement of ^{125}I-IL4.

Table 2: Binding of IL4-Cys Mutants to IL4R

protein	binding affinity[a] (relative to IL4) (%)
IL4	100
IL4C28	85
IL4C38	100
IL4C68	60
IL4C70	70
IL4C97	80
IL4C105	100

[a] Relative binding affinity was determined by comparing the calculated concentrations of IL4 and IL4-Cys mutant required for 50% displacement of ^{125}I-IL4 from Daudi or H9 cells (see Figure 5A).

To determine whether mutation of IL4 residue 28, 38, 68, 70, 97, or 105 altered receptor binding, we tested the purified cysteine mutants in ^{125}I-IL4 displacement analyses, comparing them to native IL4. Figure 5A shows the ^{125}I-IL4 bound in the presence of different concentrations of IL4 and IL4-Cys mutants. Table 2 shows the binding affinity of each IL4-Cys mutant relative to IL4, determined by comparing the concentrations required for 50% displacement of ^{125}I-IL4. Each IL4-Cys mutant bound with 60–100% the binding affinity of native IL4, indicating no significant difference in binding affinity between them. Thus, the residues mutated were probably not involved in receptor binding and the presence of a cysteine residue (which may or may not be connected to glutathione) at those positions did not significantly alter the overall IL4 structure. All IL4-Cys mutants exhibited mitogenic activity comparable to that of IL4, indicating that the mutated residues were not important for signal transduction (data not shown).

Activity of IL4-Cys Mutants Conjugated to PE35. To determine at which position in IL4 truncated PE could be attached with the least interference with receptor binding,

Table 3: Binding Affinity and Cytotoxic Activity of IL4–Toxins[a]

protein	binding affinity (%)	IC_{50} (ng/mL) DAUDI	IC_{50} (ng/mL) RAJI-TAT
IL4	100	>1000	>1000
IL4-PE38QQR	1	4.5	60
IL4C28-PE35	11	0.44	8.1
IL4C38-PE35	9	0.42	6.0
IL4C68-PE35	3.5	0.65	7.6
IL4C70-PE35	1	4.0	>100
IL4C97-PE35	0.7	45	>100
IL4C105-PE35	11	0.44	4.8

[a] Binding affinity was determined by displacement of ^{125}I-IL4 from H9 cells and results were normalized to IL4 = 100%. Cytotoxicity assays were performed by incubating Daudi or Raji-Tat cells with toxins for 20–24 h, labeling with [^{3}H]leucine, and measuring inhibition of protein synthesis. The IC_{50} is the calculated concentration necessary for 50% inhibition of protein synthesis.

PE35 was conjugated to each IL4-Cys mutant. Each IL4–PE35 conjugate consisted of a disulfide bond connecting the free cysteine of the IL4-Cys mutant and the single cysteine of PE35. IL4–PE35 conjugates appeared >95% pure by nonreducing SDS–PAGE after purification by Mono Q anion exchange (Figure 4B). Selected conjugates were monomeric when analyzed by size-exclusion chromatography (data not shown). The lack of reaction of the conjugates with DTNB confirmed the absence of free sulfhydryls in the IL4–PE35 proteins (data not shown).

We then measured the binding affinity of the IL4–PE35 toxins. Binding affinity was measured by displacement of ^{125}I-IL4 from H9 or Daudi cells. Table 3 lists the relative binding affinity of recombinant IL4–PE38QQR and the IL4–PE35 conjugates compared to IL4. Representative binding curves are depicted in Figure 5B. The data indicate that PE35 conjugated to positions 28, 38, and 105 in IL4 resulted in molecules which bound with highest affinity. IL4C28–PE35, IL4C38–PE35, and IL4C105–PE35 bound to IL4R with 9–11% the affinity of IL4, which was ~10-fold improved compared to recombinant IL4–PE38QQR (Table 3). IL4C68–PE35 bound over 3-fold better than IL4–PE38QQR. However, IL4C70–PE35, in which PE35 was conjugated to IL4 position 70, only two residues removed from position 68, bound only 1% as well as IL4 and displayed the same low binding affinity as IL4–PE38QQR. Finally, PE35 attached to residue 97 appeared to interfere with IL4R binding slightly more than IL4–PE38QQR, in which the toxin is attached to the C-terminus.

To determine if improved binding affinity resulted in improved cytotoxic activity, two different cell lines, Daudi and Raji-Tat (Puri & Aggarwal, 1992), were tested for sensitivity to IL4–PE38QQR and the IL4–PE35 conjugates. Cytotoxicity was measured by incubating cells with toxins, labeling with [^{3}H]leucine, and quantitating protein synthesis inhibition. Cytotoxicity curves are shown in Figure 6, and IC_{50}'s, the calculated toxin concentrations needed for 50% inhibition of protein synthesis, are listed in Table 3. The data show that both Daudi and Raji-Tat cells were ~10-fold more sensitive to IL4C28–PE35, IL4C38–PE35, or IL4C105–PE35 than to IL4–PE38QQR. The cytotoxicity of IL4C68–PE35 was similar to that of the three most active conjugates. In contrast, the cytotoxicity of IL4C70–PE35 was similar to or lower than that of IL4–PE38QQR. IL4C97–PE35 showed poor cytotoxic activity toward the target cells. Thus, cytotoxicity of the IL4–toxins roughly correlated with the binding affinity and confirmed that positions 28, 38, and 105 of IL4 can be connected to a toxin without severely compromising the binding affinity of IL4.

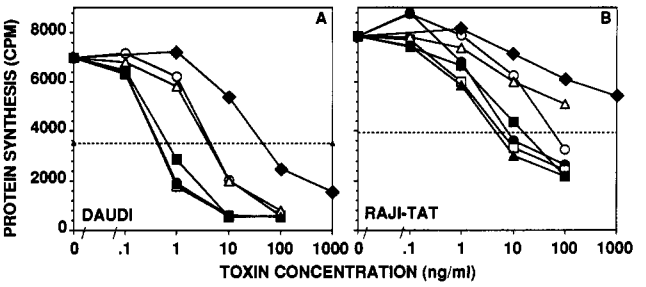

FIGURE 6: Cytotoxic activity of IL4 toxins toward Daudi (A) and Raji-Tat (B) cells. The cytotoxic activity of recombinant IL4–PE38QQR (○) was compared to that of IL4C28–PE35 (●), IL4C38–PE35 (□), IL4C68–PE35 (■), IL4C70–PE35 (△), IL4C97–PE35 (◆), and IL4C105–PE35 (▲).

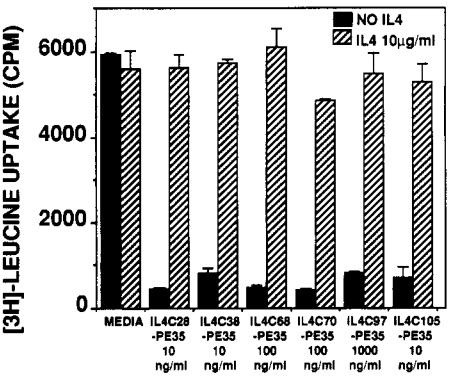

FIGURE 7: Competition of the cytotoxic activity of IL4–PE35 conjugates by excess IL4. In cytotoxicity assays, Daudi cells were incubated with the indicated concentrations of IL4–PE35 conjugates (or medium alone) with (hatched boxes) or without (solid boxes) an excess of IL4 (10 μg/mL). Error bars represent standard deviations from the means of triplicate experiments.

Cytotoxic Specificity of IL4-PE35 Conjugates. The cytotoxicity of IL4–PE^{4E} toward Daudi cells was specific for the IL4 receptor (Debinski et al., 1993). It was theoretically possible, however, that the internalization and cytotoxicity of some of the IL4–PE35 conjugates containing new ligand–toxin junctions was mediated nonspecifically or through a different receptor. To determine whether this was the case, we used an excess of IL4 to compete for the cytotoxicity of each of the IL4–PE35 conjugates. Figure 7 shows that the protein synthesis inhibition from high concentrations of each IL4–PE35 conjugate was reversed by IL4 at 10 μg/mL. Thus, both binding and cytotoxic activities of the IL4–PE35 conjugates were specific and required IL4R's.

Effect of Increasing the Spacing between the IL4 C-Terminus and the Toxin. To determine if the improvement obtained by site-specific conjugation could also be achieved in recombinant IL4–toxin by increasing the spacing between IL4 and PE, we inserted connecting peptide spacers between the C-terminus of IL4 and the toxin. Instead of the amino acids AEEASGGPE, which connect C^{129} of IL4 with G^{253} of PE in IL4–PE38QQR, we used the connectors ELK(G_4S)QAF and ELKAF(G_4S)$_3$QAF. These two new IL4–toxins, encoded by pRKL418 and pRKL428, respectively, still bound to the IL4R with 1% the affinity of IL4, constituting no improvement over IL4–PE38QQR (data not shown). To determine if it was only the ELK sequence at the C-terminus of IL4 in these constructs which interfered with binding, a mutant IL4 molecule encoded by pRKL4EL (see Materials and Methods) was made which contained only ELKA at the C-terminus of IL4. IL4-ELKA bound with ~100% the affinity of IL4 (data not shown), indicating that it was the PE40 domain of the new recombinant IL4–toxins which interfered with binding.

We conclude that toxins cannot be placed at the carboxyl terminus of IL4 without a very large decrease in binding affinity.

DISCUSSION

Our overall goal is to use IL4–toxins as potential therapy for malignant and some autoimmune disorders lacking acceptable therapy where the target cells overexpress IL4R. The goal of the present study was to improve the binding affinity and hence cytotoxic activity of IL4–toxins by conjugating truncated PE in a site-specific manner to an extra cysteine residue introduced at different sites in IL4. We found that IL4 can be engineered with an extra seventh cysteine at position 28, 38, 68, 70, 97, or 105 without significantly altering binding affinity. Furthermore, conjugation of PE35 to residues 28, 38, or 105 of IL4 resulted in IL4–toxins with 10-fold improved binding and cytotoxic activity compared to recombinant IL4–toxin.

Site-Specific Conjugation by Cysteine Mutagenesis in the Ligand. We found that cysteine mutagenesis was an efficient method to site-specifically couple the PE35 truncated toxin to the IL4 ligand, since all IL4-Cys mutants bound well (Table 2, Figure 5A) and IL4–PE35 toxins could be easily purified as 1:1 conjugates (Figure 4B). Site-specific conjugation by introduced cysteine residues has been used previously to couple a radioiodinated ligand to specific sites on the monoclonal antibody B72.3 (Lyons et al., 1990). However, nonspecific derivatization of lysine residues on antibodies and on even smaller molecules like CD4 (~40 kDa) has also allowed toxin conjugation with nearly complete preservation in binding (Pastan et al., 1992; Vitetta et al., 1987; Ghetie et al., 1990). Presumably, much of the surface of these relatively large ligands is not used for binding. For much smaller ligands such as IL4, however, sites which allow toxin conjugation with total preservation of binding may not exist, particularly if such small ligands bind to dimeric receptors. For attaching proteins to small hormones like IL4, site-specific toxin conjugation by introduced cysteine residues may be more rewarding than nonspecific conjugation.

Implications Regarding the Structure of the IL4 Receptor. The increased binding of IL4C38–PE35 compared to IL4C97–PE35 could be due either to the toxin blocking IL4R binding more at the 97 than at the 38 position or to distortion of the IL4 structure after conjugation of PE35 to residue 97. To examine if the structure of IL4 was affected more by PE35 conjugation to residue 97 than by PE35 conjugation to residue 38, we performed circular dichroism on IL4C38–PE35 and IL4C97–PE35. The θ_{215}/mg/mL values were 107, 121, and 135 for IL4C38–PE35, IL4C97–PE35, and an equimolar mixture of IL4 and PE35, respectively (data not shown). Thus, IL4 structure was not compromised more by conjugation of PE35 to residue 97 than to residue 38. Therefore IL4C97–PE35 binds to the IL4R with lower affinity than IL4C38–PE35 probably because PE35 interferes with IL4R binding more if attached to position 97 than to position 38. Information regarding which surfaces of a ligand are important for binding and which are not is potentially an important benefit of site-specific conjugation.

Several models of the unknown IL4–IL4R structure have recently been proposed. The high-resolution structure of human IL4, reported by Powers et al. (1993), suggested IL4 was structurally homologous with hGH. On the basis of the known three-dimensional structure of the hGH–hGHR homodimer complex (De Vos et al., 1992) and the assumption that the IL4–IL4R interaction might be similar, it was suggested that IL4 residues N^{15}, E^{110}, N^{111}, E^{114}, T^{118}, and R^{121} contact the first IL4R subunit while E^{9}, K^{12}, T^{13}, S^{16}, R^{85}, and R^{88} contact a second subunit (Powers et al., 1993). In another IL4–IL4R model based on the assumed structural similarity of IL4R and CD4, it was suggested that IL4 residues K^{37}, R^{115}, E^{122}, and K^{126} contact one receptor subunit, while K^{2}, K^{12}, E^{19}, Q^{20}, R^{75}, and R^{85} contact a second IL4R subunit (Bamborough et al., 1993). Our results showing that residues 28, 38, 68, 70, 97 or 105 can be mutated to cysteine without significantly altering binding (Table 2, Figure 5A) is consistent with these models.

To examine the IL4–IL4R model further in light of our IL4–PE35 data, we superimposed IL4 onto hGH in the known three-dimensional structure of hGH–hGHR, using residue equivalencies between IL4 and hGH as suggested by Powers et al. (1993). It would be expected that the greater the distance between a residue on IL4 and its nearest receptor residue, the less likely that PE35 attachment to that site would interfere with binding. Hence, if the hGH–hGHR and IL4–IL4R complexes were similar, one would expect in this IL4–hGHR model large distances between IL4 residues 28, 38, or 105 and nearest hGHR residues and smaller distances between IL4 residues 68, 70, or 97 and nearest hGHR residues. We found this was not the case, particularly with residues 97 and 105 (data not shown), indicating that the IL4–IL4R complex is structurally distinct from that of the hGH–hGHR complex. Indeed, it was recently shown that IL4 binds to a heterodimer of IL4R and IL2Rγ rather than a homodimeric receptor (Kondo et al., 1993; Russell et al., 1993).

Factors Contributing to the Decreased Binding of All Conjugates. Despite our efforts to locate a region of IL4 at which a toxin could be attached without diminishing binding activity, the best of our conjugates bound with only 11% the affinity of IL4. We believe there are several possible reasons for this decrease in binding. One is that each IL4 molecule interacts with two receptor subunits, and the 35-kDa toxin moiety, because of its large size relative to IL4, disrupts at least one of these interactions, regardless of where on IL4 it is attached. It is also possible that slight loss of IL4 structure after conjugation, documented by circular dichroism experiments on IL4C38–PE35 and IL4C97–PE35 (see above), accounts for the decreased binding affinity. A third possible reason is the kinetic effect of increasing the mass of a ligand by severalfold. It is possible that the difference in entropy change associated with binding of a large versus a small molecule to the same receptor could account for the difference in the binding affinity of IL4 and conjugates IL4C28–PE35, IL4C38–PE35, and IL4C105–PE35.

Conclusions. We have conjugated PE35 to site-specific locations in IL4 and determined that toxins containing PE35 attached to IL4 residues 28, 38, and 105 have 10-fold improved binding and cytotoxicity compared to recombinant fusions of the toxin to the IL4 C-terminus. These and related molecules will be studied further as potential treatment for IL4R-related disorders.

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