A Chimera of Interleukin 2 and a Binding Variant of Aerolysin Is Selectively Toxic to Cells Displaying the Interleukin 2 Receptor*

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Aerolysin is a bacterial toxin that binds to glycosylphosphatidylinositol-anchored proteins (GPI-AP) on mammalian cells and oligomerizes, inserting into the target membranes and forming channels that cause cell death. We have made a variant of aerolysin, R336A, that has greatly reduced the ability to bind to GPI-AP, and as a result it is only very weakly active. Fusion of interleukin 2 (IL2) to the N terminus of R336A-aerolysin results in a hybrid that has little or no activity against cells that do not have an IL2 receptor because it cannot bind to the GPI-AP on the cells. Strikingly, the presence of the IL2 moiety allows this hybrid to bind to cells displaying high affinity IL2 receptors. Once bound, the hybrid molecules form insertion-competent oligomers. Cell death occurs at picomolar concentrations of the hybrid, whereas the same cells are insensitive to much higher concentrations of R336A-aerolysin lacking the IL2 domain. The targeted channel-forming hybrid protein may have important advantages as a therapeutic agent.

The possibility that cancer cells can be selectively killed by targeted protein toxins has been explored for many years (for reviews see Refs. 1 and 2). Beginning in the 1980s, studies were conducted with protein synthesis-inactivating toxins, mainly Pseudomonas exotoxin, diphtheria toxin, and ricin, targeted to specific cell types by attaching specific antibodies or antibody fragments or by attaching growth factors such as interleukin 2 (IL2). Several of these hybrid toxins have shown some efficacy in targeted cancer treatment. One of the first of these was created by fusing IL2 to a truncated version of diphtheria toxin (3). The truncated toxin lacks the normal diphtheria toxin receptor-binding site, so that the hybrid cannot bind to normal cells. An IL2-DT hybrid, called denileukin diftitox, has been approved for treatment of patients with persistent or recurrent cutaneous T-cell lymphoma, whose malignant cells display the CD25 component of the IL2 receptor (4).

Aerolysin is a channel-forming toxin that is capable of killing mammalian cells at picomolar concentrations. The protein is secreted as an inactive protoxin called proaerolysin by Aeromonas spp. (5–7). The structure of proaerolysin has been solved, and the mechanism by which it causes cell death is well understood (8–10). Proaerolysin binds with high affinity to the glycosyl anchor of glycosylphosphatidylinositol-anchored proteins (GPI-AP), which are widely distributed on the surfaces of mammalian cells (11, 12). Surface plasmon resonance binding studies using proaerolysin variants indicate that the high affinity binding site for the ligand is located at one end of the large lobe of proaerolysin (13). Preliminary studies indicated that Arg-336 was a critical residue in binding as we found that the aerolysin variant R336A was completely unable to lyse erythrocytes.

Either before or after it has bound to the receptor, proaerolysin can be converted to aerolysin by proteolytic removal of a C-terminal fragment. This can be accomplished by a number of proteases but perhaps most importantly by furin and related proteases (14), which are transiently exposed on many mammalian cell surfaces. Aerolysin formed in solution by soluble proteases can bind to surface receptors with the same affinity as proaerolysin. Once on the cell surface, aerolysin oligomerizes to form heptamers that insert into the cell membrane, creating channels that cause cell death (15). Insertion is likely accompanied by a change in the conformation of the oligomer that allows it to penetrate and remain in the lipid bilayer (16–18).

Aerolysin may have several advantages as a component of molecules targeted to cancer cells. Production problems are likely to be minimal, as proaerolysin is a remarkably stable molecule that can be made safely in very high yield, but perhaps more importantly, the cell killing mechanism should minimize any possibility of a bystander effect. Thus once the oligomer has entered the cell membrane and killed the cell, it is unable to move to the membrane of another cell. This is because oligomer membrane entry is likely to be irreversible (19). Another advantage offered by aerolysin is that it can potentially be made specific for a cancer cell, not only by adding a specific targeting agent but also by modifying the protein so that it is activated by a protease associated with a specific cell type. We have already replaced the amino acid sequence where furin activates proaerolysin with a sequence that is cleaved by the proteolytic activity of prostate-specific antigen, creating a protoxin that is activated when injected into the prostate (20). This engineered toxin is currently the subject of a Phase I clinical trial for localized recurrent prostate cancer.

Here we provide evidence that aerolysin can be targeted to cells displaying the IL2 receptor, by first modifying the protoxin so that it does not bind to GPI-AP, which are displayed on most or all mammalian cells, and by then fusing the modified toxin to

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2 The abbreviations used are: IL2, interleukin 2; GPI-AP, glycosylphosphatidylinositol-anchored protein(s); IL4, interleukin 4; GP1-A; glycophosphatidylinositol-anchored protein(s); PA, proaerolysin; uPA, urokinase-type plasminogen activator; BisTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; PBS, HEPES-buffered saline; SS, signal sequence; MOPS, 4-morpholinepropanesulfonic acid; fwd, forward; rev, reverse; h, human.
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**TABLE 1**

| Strain | Plasmid | Description | Source |
|--------|---------|-------------|--------|
| E. coli DH5α | pTZ18U | Cloning vector, Ap<sup>+</sup> | Bio-Rad |
| E. coli DH5α | pMMB66HE | Broad host range expression vector, Ap<sup>+</sup> | Furste et al. (22) |
| E. coli | pPA-H6 | aera gene with His tag attached to its 3′-end in cloning vector pTZ18U | This study |
| E. coli | pPA-H6-MMB | aera gene with His tag attached to its 3′-end in expression vector pMMB66HE | This study |
| E. coli | pNII2 | Gene for human IL2 with codons for G27, G88 (GGG) changed to GGC in vector pGEM1 | This study |
| E. coli | pIL2 | IL2-uPA-PA-H6 variant, in cloning vector pTZ18U | This study |
| E. coli | pIL2-MMB | IL2-uPA-PA-H6 variant, in expression vector pMMB66HE | This study |
| A. salmonicida CB3 | pR336A | aera gene with His tag attached to its 3′-end with the R336A mutation in cloning vector pTZ18U | This study |
| A. salmonicida CB3 | pR336A-MMB | aera gene with His tag attached to its 3′-end with the R336A mutation in expression vector pMMB66HE | This study |
| A. salmonicida CB3 | pIL2-R336A | IL2-uPA-PA-H6 variant with the R336A mutation, in cloning vector pTZ18U | This study |
| A. salmonicida CB3 | pIL2-R336A-MMB | aera gene with His tag attached to its 3′-end with the R336A mutation in expression vector pMMB66HE | This study |

*Experimental Procedures*

**Reagents—**Media for the propagation of mammalian cell lines, Taq DNA polymerase, T4 DNA ligase, and primers were from Invitrogen; T-STEM with concanavalin A was from BD Biosciences; restriction nucleases and Deep Vent DNA polymerase were from New England Biolabs (Beverly, MA); QuikChange II site-directed mutagenesis kits were from Stratagene (La Jolla, CA); and QIAexpress gel extraction kits (for purification of fragments from agarose gels) and QIAprep spin miniprep kits (for plasmid purification) were from Qiagen (Mississauga, Ontario, Canada).

**Cell Lines—**All cell lines were from the American Type Culture Collection (Manassas, VA). The CTLL-2 mouse cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10% T-STEM with concanavalin A or with 100 units/ml of human recombinant IL2. The EL-4 mouse cell line was maintained in Dulbecco’s modified Eagle’s medium supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum. The HuT-78 human cell line was maintained in Iscove’s modified Dulbecco’s medium supplemented with 20% fetal bovine serum. The Ramos and IARC 301 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. Sodium pyruvate was also present in the Ramos growth medium.

**Bacterial Strains and Plasmids—**The bacterial strains and plasmids are listed in Table 1. *Escherichia coli* was normally grown in LB media containing the appropriate antibiotics at 37 °C. *Aeromonas salmonicida* was grown in LB media supplemented with 0.2% (w/v) glucose and Davis salts (21) at 27 °C.

**Plasmid Construction—**All plasmids used in this study were propagated in *E. coli* DH5α (unless stated otherwise). The gene for PA encoding six histidines at the C terminus of the protein was prepared by inserting six codons (CAT) at the 3′-end of the gene by two rounds of site-directed mutagenesis using a QuikChange II site-directed mutagenesis kit, two pairs of primers (EndHis1fwd, GCTGCGCAGGGCAAGTATCATCATTAAACGGCAGCGC; and EndHis1rev, GCCCGTGGATTAATAATTACAAGAATCC), and Plasmid Analyzer, Cloning vector, Ap<sup>+</sup> | Bio-Rad |

**Results and Discussion—**The resulting hybrid is a new kind of targeted toxin that may be useful in cancer therapy.

**Conclusion—**To accommodate the codon usage for *Aeromonas* sp., two GGA codons for Gly (Gly-27 and Gly-88) in the gene coding for IL2 were changed to GGC, which is preferred by *Aeromonas*. Two rounds of site-directed mutagenesis were performed using a QuikChange II site-directed mutagenesis kit, two pairs of primers (Gly-27fwd, CTGGATTTACAGATGATTGGCAGC; Gly-88fwd, GGTCTTGTGATATTAATGAGCTC; and Gly-27rev, GTTTCTGAGACTAAGGGGGTCTGAGTC; and Gly-88rev, TTACACATGAGTTGGTTCTCAGGCCCCTAGTTCCGAGAC) and plasmid pGEM1 containing the gene for human interleukin 2 (hIL2) as a template according to the manufacturer’s instructions. The resulting plasmid was named pNII2.

**Aeromonas—**The DNA fragment containing the gene for hIL2 (amino acids 2–131), with a sequence encoding the 3′-end of the signal sequence (SS) of aerolysin at the 5′-end and a sequence encoding a six amino acid linker (SGRSAQ), which could be cleaved by uPA at the 3′-end, was prepared by PCR1 using the primers PSLfwd, CAAGCGCCAGGCGCTATTCAAGTTT; and UPARrev, TTACACATGAGTTGGTTCTCAGGCCCCTAGTTCCGAGAC; and plasmid pGEM1 containing the gene for human interleukin 2 (hIL2) at the 3′-end was prepared by PCR2 using the primers pUCfwd, GGCTGCGCAGGGCAAGTATCATCATTAAACGGCAGCGC; and EndHis2rev, GCCCTGCGCAGGGCAAGTATCATCATTAAACGGCAGCGC, and plasmid pNII2 as a template. The DNA fragment containing the sequence for the SS of aerolysin with the part of hIL2 gene at the 3′-end was prepared by PCR3 using the primers pUCfwd, GGCTGCGCAGGGCAAGTATCATCATTAAACGGCAGCGC; and EndHis2rev, GCCCTGCGCAGGGCAAGTATCATCATTAAACGGCAGCGC, and plasmid pNII2 as a template. The DNA fragment containing the sequence for the SS of aerolysin with the part of hIL2 gene at the 3′-end was prepared by PCR4 using the primers pUCfwd, GGCTGCGCAGGGCAAGTATCATCATTAAACGGCAGCGC; and EndHis2rev, GCCCTGCGCAGGGCAAGTATCATCATTAAACGGCAGCGC, and plasmid pNII2 as a template. The DNA fragment containing the sequence for the SS of aerolysin with the part of hIL2 gene at the 3′-end was prepared by PCR5 using the primers pUCfwd, GGCTGCGCAGGGCAAGTATCATCATTAAACGGCAGCGC; and EndHis2rev, GCCCTGCGCAGGGCAAGTATCATCATTAAACGGCAGCGC, and plasmid pNII2 as a template.

**Conclusions—**The resulting hybrid is a new kind of targeted toxin that may be useful in cancer therapy.
the plasmid pPA-H6 as a template. The whole recombinant gene SS-IL2-uPA-PA-H6 was prepared by recombinant PCR (PCR5) with the primers uPCfw and uPCRv, using the products of PCR3 and PCR4 as a template.

The product of PCR5 was cleaved with the restriction endonucleases HindIII and EcoRI and cloned into the cloning vector pTZ18U cut with the same enzymes. After the sequence of the gene was confirmed by DNA sequencing, the gene was cloned into the broad host range expression vector pMMB66HE (22) using HindIII and EcoRI and moved into A. salmonicida CB3 by conjugation using the filter-mating technique described by Harayama et al. (23). DNA manipulations were carried out as described by Sambrook et al. (24).

Mutagenesis—The R336A variants of the proteins were prepared by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit with the pair of primers R336Afwd, CACCCGGACACGCGACCGACTGAAC-CAC, and R336Arev, GTGGTTCCAGTGGTGCCTG-GTCGGGGTG, and the corresponding wild type PA gene cloned in the vector pTZ18U as a template. After the sequence of the mutated gene had been confirmed by DNA sequencing, the gene was cloned into the broad host range expression vector pMMB66HE (using HindIII and EcoRI) and moved into A. salmonicida CB3 by conjugation using the filter-mating technique (23).

Expression and Purification of Proaerolysin—Both native proaerolysin and R336A proaerolysin were expressed in A. salmonicida CB3 and purified from the bacterial culture supernatant as described previously (25).

Expression and Purification of IL2 Hybrids—The two IL2 hybrids were expressed in 400 ml of CB3 II-PA-uPA-PA-H6::66 or CB3 II-PA-R336A-H6::66 (starting A400 nm 0.7, final A400 nm 5–6) by 1 ml isopropyl-D-thio-β-D-galactopyranoside induction followed by shaking overnight at 250 rpm and 27 °C. The cells were centrifuged at 10,000 rpm for 10 min at 4 °C and resuspended in 40 ml of phosphate-buffered saline containing 10 μg/ml DNase, 10 μg/ml RNase, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. The IL2 hybrid was released from the cells by French pressing 3–4 times at 800 p.s.i. to form a cell lysate that was clarified by centrifugation at 10,000 rpm, 10 min, 4 °C. Half of the clarified cell lysate was separated by fast protein liquid chromatography nickel affinity chromatography using a 10-ml chelating-Sepharose Fast Flow (Amersham Biosciences) column at room temperature. The fast protein liquid chromatography program used two buffers as follows: buffer A, 20 mM NaHPO4, 500 mM NaCl, 10 mM imidazole, pH 7.4; and buffer B, 20 mM Na2HPO4, 500 mM NaCl, 500 mM imidazole, pH 7.4. The cell lysate was loaded onto the column equilibrated in buffer A, and the column was washed with 100 ml of 10% buffer B (50 mM imidazole, pH 7.4). Elution was achieved using a gradient of 10–60% buffer B. The peak fractions were pooled and loaded onto a 60-ml macro-prep ceramic hydroxyapatite type 1 40-μm column (Bio-Rad) equilibrated at 4 °C in 20 mM KH2PO4, 300 mM NaCl, pH 7.4. Each of the IL2 hybrids was eluted using a linear gradient of 20–200 mM KH2PO4 in 300 mM NaCl, pH 7.4. Protein concentrations were estimated by absorbance at 280 nm.

Hemolytic Titers—Hemolytic titers were performed at 37 °C using a final concentration of 0.4% (v/v) horse red blood cells in HBS. The toxin samples (3.5 μg) were preincubated with one of the following proteases: 20 units/ml furin (Sigma) in HBS, 10 mM CaCl2, pH 7.4, for 4 h at 37 °C; 0.04 mg/ml urokinase plasminogen activator (uPA) in HBS for 4 h, or 0.1 mg/ml trypsin in HBS for 10 min at room temperature. Controls were incubated in HBS for 10 min at room temperature without the addition of protease. All digestions were stopped by the addition of phenylmethylsulfonyl fluoride (1 mM final concentration) and cooled on ice. For each proaerolysin variant, a volume of reaction mixture containing 3 μg of toxin was transferred to the well in the first column of a 96-well titer plate (two plates were used). The volume of the well was brought to 200 μl with HBS. Each sample was serially diluted 1:1 across the plate. Readings were taken after 1 h at 37 °C. Results are expressed in units representing the largest dilution causing complete cell lysis.

Cell Killing Assays—Killing assays were carried out using cell lines prepared at concentrations of 1 × 106 cells/ml. Each assay was carried out in triplicate. Using 96-well titer plates, each of the proteins was serially diluted 1:5 in supplemented cell media using a final volume of 20 μl. After serial dilution, cells were added to appropriate rows to a final volume of 100 μl. Two control rows were used; the first contained no toxin and no cells, and the second contained no toxin with cells. The cells were then preincubated with the toxins for 1 h at 37 °C, under 5% CO2 with humidity before adding 20 μl of cell killing reagent (Promega CellTiter 96 AQueous One Solution cell proliferation assay). This was followed by a further incubation for 4 h under the same conditions. The plates were then read using a BioTec plate reader at 490 nm. Cell killing curves were generated by plotting the calculated average percent cell viability for each well against the respective toxin concentration. For IL4 replacement cell killing assays, 200 units/ml mouse IL4 (BioVision) were added to each well prior to toxin dilutions. Similarly, human recombinant IL2 was added to a final concentration of 10 μg/ml for IL2 competition cell killing assays.

Flow Cytometry—Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences). EL4 and CTLL-2 cells were prepared in unsupplemented cell culture media to a concentration of 2 × 106 cells/ml. Toxin was added to cells at 2 × 10−8 M, and the mixtures were incubated on ice for 30 min. Cells were washed three times in unsupplemented media and resuspended to the original volume. Rabbit anti-aerolysin polyclonal antiserum was added at a 1:500 dilution, and each mixture was incubated on ice for 30 min, and then the cells were washed and resuspended as above. The secondary antibody, a polyclonal goat α-rabbit IgG H+L chain-specific fluorescein conjugate (Calbiochem), was added at a 1:5000 dilution, and the mixtures were incubated on ice for 30 min. Cells were washed and resuspended once again as above and readings were taken. Histograms were prepared using Cellquest software.

Oligomerization Study—The oligomerization study was performed by incubating PA or R336A at 0.08 mg/ml with 0.002 mg/ml trypsin for 20 min at 37 °C. Trypsin inhibitor was then added to 0.1 mg/ml, and incubation was continued for 1 h. Samples in 1X Sample Buffer (NuPAGE) were loaded onto a
NuPAGE 10% BisTris gel (Invitrogen) in 1× MOPS-SDS running buffer under nonreduced conditions and run for 50 min at 200 V constant voltage. The gel was stained with Coomassie Brilliant Blue R-250.

The cell killing oligomerization study was performed by incubating CTLL-2 cells in cell culture media with each of the specified proaerolysin constructs for 5 h. After incubation, the cells were pelleted and resuspended in sample buffer (NuPAGE), and the samples were electrophoresed using 10% BisTris NuPAGE gels (Invitrogen) in 1× MOPS-SDS buffer under nonreduced conditions. Aerolysin monomers and oligomers were detected by Western blotting using an anti-aerolysin monoclonal antibody.

RESULTS

Purification of PA and Hybrid Variants—Proaerolysin and R336A-proaerolysin (R336A-PA) were both secreted into the culture supernatant of A. salmonicida, and both were purified as we have described previously. Neither of the IL2 containing hybrids was secreted by the bacteria, presumably because the IL2 moiety prevented transfer of the proteins across the outer membrane. The two hybrid proteins were purified from the soluble fraction after French pressing and centrifugation. All of the purified proteins migrated as single major bands of expected apparent mass upon SDS-PAGE (Fig. 1).

Binding of R336A-PA to Lymphocytes—We have previously shown using flow cytometry that native proaerolysin binds well to EL4 cells, which display GPI-anchored Thy-1 in high copy number, as well as other GPI-anchored proteins (26). Residue Arg-336 is in a region that we have shown is involved in proaerolysin binding to GPI-AP. The results in Fig. 2 compare the binding of native proaerolysin and the R336A variant to EL4 cells. As predicted from our earlier studies on proaerolysin variants containing mutations in the same region of the molecule (13), and consistent with our observation that the variant was inactive against erythrocytes, R336A-PA was not able to bind to EL4 cells.

Proteolytic Processing of PA and Hybrid Variants—Native proaerolysin is converted to aerolysin by proteolytic removal of ~40 residues from the C terminus (the actual number removed depends on the protease used). Once formed, aerolysin is resistant to further proteolysis (27). The results in Fig. 3 show that both PA and R336-PA were converted to a form corresponding in mass to aerolysin by treatment with trypsin, uPA, or furin. This was not surprising because the binding variant and PA have the same amino acid sequence in the activation loop. Trypsin and uPA also converted IL2-PA and IL2-R336A-PA to aerolysin, producing a band corresponding in mass to IL2 in the process. Thus both of these enzymes could cut at the six amino acid linker joining IL2 to PA, as well as within the PA C-terminal activation sequence. In contrast, furin converted the IL2 proaerolysin hybrids each to the IL2-aerolysin form but did not...
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**TABLE 2**

| Hemolytic titers of PA constructs | None | Trypsin | Furin | Urokinase |
|-------------------------------|------|---------|-------|-----------|
| PA                            | 0    | 128     | 128   | 64        |
| R336A-PA                      | 0    | 4       | 4     | 4         |
| IL2-uPA-PA                    | 0    | 32      | 4     | 8         |
| IL2-uPA-R336A-PA              | 0    | 2       | 0     | 0         |

Hemolytic titers were performed at 37°C using a final concentration of 0.4% (v/v) horse red blood cells in HBS. The toxin samples were pretreated with furin, uPA, or trypsin and titered as described under “Experimental Procedures.”

**FIGURE 4. Oligomerization of PA and R336A-PA.** Native PA (lanes 1 and 3) and R336A-PA (lanes 2 and 4) were treated with trypsin (lanes 1 and 2) as described under “Experimental Procedures,” and the formation of oligomers (O) was analyzed by SDS-PAGE. (PA, praerolysin; A, aerolysin.)

separate the IL2 from the aerolysin. This was not surprising, because the linker peptide between the two parts of the hybrid does not contain a sequence recognized by furin. There was no evidence that the IL2 was degraded by any of these proteases, before or after it was released from the hybrids, indicating that it was correctly folded.

**Hemolytic Activity of Processed PA and Hybrid Variants—** Mammalian erythrocytes lack proteases that can convert praerolysin to aerolysin, and as a result they are unaffected by the protoxin. The results in Table 2 show that treatment of native PA with trypsin, furin, or uPA converted it to hemolytically active aerolysin, consistent with the results in Fig. 5. The results in Table 2 also show that R336A-aerolysin, formed from R336A-PA by any of the three proteases or from IL2-R336A-PA by uPA or trypsin, was incapable of lysing erythrocytes, presumably because it cannot bind to the cells. The results in Table 2 also show that IL2-aerolysin, produced by treatment of IL2-PA with furin (Fig. 3), had some ability to lyse erythrocytes. This indicates that the presence of the IL2 moiety does not prevent the formation of a channel-forming oligomer.

**Oligomerization of R336A-PA and Native PA—** We next considered the possibility that the R336A mutation in praerolysin might not only prevent the protein from binding to GPI-AP but might also prevent R336A-aerolysin from oligomerizing. Oligomers normally form on the target cell surface, after praerolysin is concentrated there by receptor binding and converted to aerolysin by proteolytic nicking. It is the oligomeric form of the toxin that is insertion-competent and that forms membrane channels (15). Aerolysin oligomers are very stable, and they can be detected by SDS-PAGE. To investigate the possibility that the R336A mutation affected the ability of the protein to oligomerize as well as to bind, we compared the ability of the R336A-PA variant to oligomerize with the oligomerization ability of native praerolysin using a method we have described previously (15). Briefly, the protein at a high concentration was converted to the aerolysin form by trypsin; trypsin inhibitor was added, and incubation was continued to allow oligomerization to occur. The oligomers were then detected by SDS-PAGE. The results in Fig. 4 show that native PA and R336A-PA formed similar amounts of oligomer, confirming that the variant is able to oligomerize.

**Binding to Mammalian Cells—** We next measured binding of our constructs to five cell lines using flow cytometry. Constructs that contain the native GPI-anchor-binding site of praerolysin should bind to cells displaying these receptors, and constructs with the IL2 domain should bind to cells with high affinity IL2 receptor complexes. High affinity binding of IL2 requires the presence of both the α and β subunits of the receptor. All of the cell types we tested display GPI-AP on their surfaces, but they vary in the types and numbers of the IL2 receptor subunits they display. Unactivated EL4 cells do not have IL2 receptor α subunits (28, 29), and HuT 78 cells have fewer than 20 of these subunits per cell (30). Ramos cells have large numbers of α subunits but no β subunits (31), whereas IARC 301 cells have relatively small numbers of both (32). Of the tested cells, only the CTLL-2 cells have large numbers of both the α and β subunits (4). The results in Fig. 5 show that, as expected, native PA could bind to all of the cell lines. The IL2-PA variant could also bind to all of the cells we tested, but more poorly than native PA, perhaps because the IL2 domain interfered with the GPI-AP-binding site in the PA component. The R336A-PA could not bind to any of the cell lines, consistent with destruction of the GPI-AP-binding site. The IL2-R336A-PA showed no binding to EL4 cells, which lack IL2 receptors and only a small amount of binding to HuT 78, IARC 301, and Ramos cells, each of which has only a small number of high affinity receptors. The IL2-R336A-PA hybrid bound very well to CTLL-2 cells, consistent with the presence of large numbers of high affinity IL2 receptors on these cells. Hence the results indicate that the IL2 component of the hybrid is capable of interaction with its receptor, bringing the molecules to the cell surface.

**Cell Killing by PA and Hybrid Variants—** We next carried out cell killing assays with PA, R336A-PA, and the corresponding IL2 hybrids, using the same cell lines as above. The results are shown in Fig. 6. As expected, native praerolysin was active against all of the cell lines, consistent with the binding data in Fig. 5. In contrast, the R336A-PA variant was inactive or only very weakly active against all of the cells, likely because of its reduced ability to bind to GPI-AP. The IL2-PA was also active against all cell lines, indicating that these cells are capable of binding the hybrid via GPI-anchored proteins (or also via the IL2 receptor in the case of CTLL-2 cells). The results in Fig. 6 show that IL2-R336A-PA was inactive or weakly active against...
all of the cells that have few or none of the high affinity IL2 receptor molecules, but it was capable of killing CTLL-2 cells at nanomolar concentrations. Similar results were obtained when IL4 was added to the medium, indicating that the effect of IL2-R336A-PA on the CTLL-2 cells was not because of the absence of IL2 in the medium (not shown here), as these cells can live without IL2 if IL4 is added to the medium (33). Furthermore, addition of IL2 to the medium reduced CTLL-2 killing by IL2-R336A-PA, evidence that the cytokine was competing with the hybrid proaerolysin variant for receptor sites on the cell surface (Fig. 7).

**Oligomerization Accompanying Cell Killing**—The results of the CTLL-2 killing assay indicate that IL2-PA and IL2-R336A-PA are capable of killing the cells, whereas the R336A-PA variant is not. Killing by channel formation will only occur if the toxin binds to the cells, is activated, and forms oligomers. We next tried to determine whether oligomers were formed during exposure of CTLL-2 cells to IL2-PA, IL2-R336A-PA, and R336A-PA. Much lower protein concentrations were used in this experiment than in the experiment depicted in Fig. 4. Under these conditions aerolysin concentrations were not high enough for oligomerization to occur in solution. Binding to the cell surface was necessary to raise the aerolysin concentration high enough to promote oligomerization. The results in Fig. 8 show that oligomer was easily detected after cells were exposed to native proaerolysin or to either of the hybrids. The oligomer formed by the IL2 hybrids was larger than the oligomer formed by native aerolysin, indicating that the cells did not cut the uPA linker and remove the IL2, and also that it is the hybrid oligomers that are responsible for channel formation and cell death. Little or none of the proaerolysin and aerolysin forms of the R336A-PA variant were found associated with the cells, and no oligomer was detected after exposure to R336A-PA. This result was not surprising because the results in Fig. 5 had shown that R336A-PA cannot bind to the cells.

**DISCUSSION**

There is an urgent need to develop agents that are both efficacious and safe for cancer therapy. The results of this study illustrate the potential advantages of the channel-forming protein aerolysin as a component of a targeted toxin hybrid. Aerolysin and its inactive precursor, proaerolysin, both bind with high affinity to GPI-AP on the surfaces of mammalian cells. To target the toxin to specific cells, we first altered the GPI-AP-binding site on
the protein to prevent it from binding nondiscriminately. The proaerolysin variant we constructed, R336A-PA, was inactive or far less active than native proaerolysin against all the cell types we tested, although we showed that it was converted to the aerolysin form by proteolysis and that once converted it could oligomerize in the same way as native toxin. Flow cytometry and Western blotting confirmed that R336A-PA could not bind to cells, accounting for its inactivity.

Having made a proaerolysin variant that has greatly reduced ability to bind to mammalian cells, we then attempted to target the protoxin to cells displaying the IL2 receptor by fusing IL2 to the N terminus of R336A-PA. We made a similar fusion to native proaerolysin, and we used this hybrid to show that the addition of IL2 did not destroy the ability of aerolysin to kill mammalian cells. We showed that the presence of the cytokine attached to the N terminus of aerolysin did not prevent oligomerization, which is thought to involve a loop in domain 3 of the toxin. Oligomers could be detected by Western blotting, and they were capable of insertion and channel formation, as they caused erythrocyte lysis.

The addition of IL2 to the N terminus of proaerolysin did however block secretion of the hybrid by A. salmonicida. Native proaerolysin is secreted into the culture supernatant of this organism by the main terminal branch of the type II secretory pathway. The protein first crosses the inner bacterial membrane in an unfolded state and then obtains its native conformation in the periplasm before crossing the outer membrane (5). This latter step is known to depend on the structure of the secreted molecule and presumably the presence of IL2 alters the structure so the protein cannot cross the membrane. The hybrid proteins were able to cross the inner membrane of both A. salmonicida and E. coli, and we purified both hybrids from the latter species. Both the IL2 and the aerolysin components of the hybrids appeared to be correctly folded, as they were resistant to proteolysis, and they retained their separate functions.

The IL2-R336A-PA hybrid was inactive against all of the cells we tested except CTTL-2 cells, which display large quantities of the three subunits of the IL2 receptor (4). Hut 78 lymphocytes, which contain fewer than 20 molecules per cell of the p55 subunit of the receptor (4), were not affected by this hybrid, nor were IARC 301 cells or Ramos cells. Thus the fusion of the IL2 molecule to R336A-PA had the desired outcome of targeting the toxin to specific cells that overexpress the IL2 receptor. The fact that IL2-R336A-PA bound much more poorly to the Ramos cells than to CTTL-2 cells, and the observation that Ramos cells were much less sensitive to this hybrid than were CTTL-2 cells, indicates that, as with IL2 itself, the binding of this hybrid is dependent on the presence of high affinity IL2 receptors, which require both α and β subunits of the receptor.

Aerolysin has two important advantages as a candidate for targeted toxin therapy. First, the toxin is single hit, that is it is not capable of moving from cell to cell; and second, it can be targeted to specific cells in at least two ways. The first level of targeting takes advantage of the fact that proaerolysin must be activated by proteolytic removal of a C-terminal peptide before it can form a channel. Activation of native proaerolysin is probably accomplished on the cell surface by the enzyme furin; however, the region where the protein is cleaved can be altered, removing furin activation and replacing it with a sequence that can be cleaved by another protease. We have incorporated a number of different activation sequences into proaerolysin, including a sequence that is cleaved by prostate-specific antigen. The resulting proaerolysin, PSA-PAH1 is currently the subject of a Phase I clinical trial for the treatment of localized recurrent prostate cancer. The results of this study show that aerolysin can also be targeted in another way, by adding a specific binding moiety such as IL2. The fusion protein bound selectively to cells displaying high affinity IL2 receptors, and it was correctly processed to the aerolysin form. The activated hybrid then oligomerized in the same way as native aerolysin, and this led to cell death. Future proaerolysin molecules, targeted to specific cells by adding a binding subunit as well as by incorporating a selective activation sequence, may represent an important new approach to cancer therapy.

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