Ligand-toxin Hybrids Directed to the $\alpha_2$-Macroglobulin Receptor/Low Density Lipoprotein Receptor-related Protein Exhibit Lower Toxicity than Native Pseudomonas Exotoxin*

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Pseudomonas exotoxin (PE) binds the heavy chain of the $\alpha_2$-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP). To understand the significance of this interaction, novel toxin-derived gene fusions were constructed with two ligands that also bind this receptor. A 39-kDa cellular protein, termed RAP, binds LRP with high affinity and often co-purifies with it. Two RAP toxins were constructed, one with PE and one with diphtheria toxin (DT). DT-plasminogen activator (PAI)-1, which replaced the toxins binding domains, was combined with each of the corresponding translocating and ADP-ribosylating domains. Both RAP-toxins bound LRP with an apparent higher affinity than native PE. Despite this, RAP-PE and DT-RAP were less toxic than native PE. Apparently, RAP-toxin molecules bound and entered cells but used a pathway that afforded only low efficiency of toxin transport to the cytosol. This was evident because co-internalization with adenovirus increased the toxicity of RAP-toxins by 10-fold. We speculate that the high affinity of RAP binding may not allow the toxin’s translocating and ADP-ribosylating domains to reach the cytosol but rather causes the toxin to take another pathway, possibly one that leads to lysosomes. To test this hypothesis, additional RAP-PE fusions were constructed. N-terminal or C-terminal fragments of RAP were joined to PE to produce two novel fusion proteins which were likely to have reduced affinity for LRP. Both of these shorter fusion proteins exhibited greater toxicity than full-length RAP-PE. A second ligand-toxin gene fusion was constructed between plasminogen activator inhibitor type 1 and DT. DT-plasminogen activator inhibitor type 1 formed a complex with tissue-type plasminogen activator and inhibited its proteolytic activity. However, like the RAP-toxins, this hybrid was less toxic for cells than native PE.

The $\alpha_2$-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP) is one of the largest membrane-associated proteins characterized to date. Its primary amino acid sequence was derived from overlapping cDNA clones (1).

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§ The abbreviations used are: LRP, $\alpha_2$-macroglobulin receptor/low density lipoprotein receptor-related protein; DT, diphtheria toxin; ER, endoplasmic reticulum; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; PAI-1, plasminogen activator inhibitor type 1; PE, Pseudomonas exotoxin; RAP, receptor-associated protein; tPA, tissue-type plasminogen activator; PAGE, polyacrylamide gel electrophoresis.

LRP is located both on surface and intracellular membranes of many eukaryotic cell types. It is synthesized as a 4525-amino acid single chain precursor. After synthesis, possibly in the Golgi, single chain LRP is cleaved to give a 515-kDa heavy chain and an 85-kDa light chain (2). The light chain contains both a membrane-spanning region and cytoplasmic tail. The heavy chain which remains membrane-associated via non-covalent interactions with the light chain, contains the ligand-binding sites. LRP mediates the binding and endocytosis of several unrelated ligands: 1) apoprotein E-enriched $\beta$-migrating very low density lipoprotein (3, 4); 2) peptidase- or methylamine-activated $\alpha_2$-macroglobulin (5); 3) the complex between tissue-type plasminogen activator (tPA) or urokinase with plasminogen activator inhibitor type 1 (PAI-1) (6, 7). A 39-kDa receptor-associated protein, called RAP, also binds to LRP (8, 9). Recently, it was shown that RAP has two regions that can independently, although less efficiently, interact with LRP and another protein from the same family: gp330 (10, 11, 12). RAP inhibits the binding and or uptake of all known ligands that interact with LRP. At a minimum, RAP binds LRP with high affinity at two sites and possibly causes a conformational change in receptor structure. The addition of either heparin or $\beta$-mercaptoethanol inhibits the interaction of RAP with LRP (13). Recently, LRP was proposed as the cell surface receptor for Pseudomonas exotoxin (PE) (14–16).

Both PE and diphtheria toxin (DT) inactivate the protein synthetic apparatus of eukaryotic cells in a series of steps that include: binding to a surface receptor, endocytic uptake, cell-mediated proteolytic processing to generate an enzymatically active fragment, translocation of the fragment to the cell cytosol, and ADP-ribosylation of elongation factor 2.

PE is synthesized as a single chain bacterial protein composed of three structural domains. The N-terminal domain mediates binding to LRP: specifically, Lys-57 and possibly other nearby residues mediate binding (17). Toxicity for cells is reduced by at least 100-fold when Lys-57 is changed to glutamic acid or when most or all of this domain is deleted. The middle domain of PE has two functions: it contains sequences necessary for translocation to the cell cytosol and it serves as a substrate for cell-mediated cleavage (18, 19). The C-terminal domain has the ADP-ribosylating activity and contains a putative endoplasmic reticulum (ER) retention sequence (20–22). Functionally DT is quite similar to PE. However, the binding and ADP-ribosylating domains of DT are located in the opposite orientation to PE, i.e., the binding domain of DT is at the C terminus (23) while the ADP-ribosylating activity is at the N terminus (24). Like PE, the middle domain of DT contains sequences that mediate translocation to the cell cytosol (25). While the exact intracellular location for the translocation of either toxin has not been clearly defined, existing data suggests
that the A chain of DT reaches the cytosol from an acidic endosomal compartment while the corresponding PE fragment needs to reach the ER to facilitate its translocation. Thus it appears that these functionally similar toxins use different intracellular pathways to reach the same cytosolic location.

By replacing the binding domains of either PE or DT with binding ligands of various specificities it has been possible to redirect toxic activity to cells bearing particular cell surface receptors (26). For instance, DT and PE have been targeted to IL-2, IL-4, IL-6, and epidermal growth factor receptors. And although some losses in binding affinities has been noted, it is possible to produce active hybrid toxins by placing a particular ligand either at the N terminus of PE or the C terminus of DT.

As a way to study both intracellular receptor traffic and possible differences in toxin processing, we have constructed a number of ligand-toxin hybrids which bind to LRP. Because ligands will interact with different portions of the same receptor and possibly bind with different affinities, it may be possible to determine which interactions lead to efficient delivery of active toxin fragments to the cell cytosol and which do not.

To create hybrid molecules with cytotoxic characteristics that can be compared with native PE, we have replaced the receptor-binding domains of DT and PE with either RAP or tPA-PAI-1. Results indicated that these hybrid proteins exhibited much lower cytotoxicity than native PE. We also produced RAP-PE fusions composed of RAP fragments in place of full-length RAP. These proteins were more active than toxin fusion made with whole RAP. Based on these data we speculate that RAP- and tPA-PAI-1-containing recombinant proteins possess lower cytotoxicity than PE because they are less capable of dissociating from LRP and therefore are transported to lysosomes more readily than PE.

MATERIALS AND METHODS

Plasmids—The cDNA for RAP was encoded on the plasmid pRAP1-23 (27); plasmid pET-tPA-10-6 is a derivative of plasmid pET3b (28) and contains a gene encoding a fragment of the mouse a2-macroglobulin receptor; plasmids pSAU8 (26), pLABA7 (29), pAPA4 (30), and pVCDT1-1-L2 (31) have been described earlier. Plasmids were propagated in Escherichia coli DH5a (Life Technologies, Inc.). For expression of hybrid proteins, plasmids were transformed into E. coli BL21(DE3) (28).

Restriction Enzymes—EcoRI, Scal, Stul, SalGI, BamHI, Smal, Xbal, NtdI, Kpnl, as well as T4 DNA polymerase, calf intestinal phosphatase, and T4 DNA ligase were from Boehringer Mannheim; Taq DNA polymerase was from Perkin Elmer; First-strand cDNA synthesis kit was from Pharmacia Biotech Inc.

Oligonucleotides—The following oligonucleotides were from BioServe Biotechnologies and were used for polymerase chain reaction amplification: 5’-TCCAGGTCCGGCCGCGGTGGGGAAGAA-GCCGC (rap-NtdI) and 5’-TTTTTTTTTTTGACCGGCGTTCGT-GGCGAGCTCT (rap-Kpnl) were used to amplify a cDNA sequence encoding whole RAP, using pRAP1-23 as a template while rap-NtdI and 5’-TTTTTTTTTTTGACAGGGGGAAGAGTAGC (rap-Kpnl) were used to amplify a cDNA sequence encoding the N-terminal portion of RAP (amino acids from 1 to 175) and 5’-AAAAA-AAGGCTTCGGGGCCAGACCTGAGGAGGTTATGAC (rap-Kpnl) were used to amplify a cDNA sequence encoding the C-terminal portion of RAP (amino acids from 176 to 323). 5’-TTGTTGTAAGGGGTCTGCGACATCCCCCATCT and 5’-CCGAGGTACCGGTTTACTATGCCC were used to amplify the sequence encoding PAI-1.

Reagents—tPA was a gift from Dr. Uli Brinkmann, NIH. N-Methylsulfonflyl-o-Phe-Gly-Arg-4-nitroanilide acetate was from Boehringer Mannheim. Polyclonal rabbit antibodies against the heavy chain of LRP were prepared as described (32), polyvalent horse antibodies against DT conjugated with horseradish peroxidase were a gift from Dr. Smirnov (Russia). Human liver poly(A) RNA was from Clontech. Heparin was from Sigma. Heparinase was from U. S. Biochemical Corp.

Expression and Purification of Recombinant Proteins—At an absorbance of 0.5–0.6 (590 nm), protein expression was induced by the addition of isopropyl-b-D-thiogalactoside. Cells were harvested 90 min later. DT-PAK-RAP was recovered from the periplasm of E. coli by osmotic lysis. Although RAP-PE hybrids were found in a soluble form in the cytoplasm, in the presence of rifampin most of the proteins were insoluble. The use of DT-PAI-1 was hampered by its propensity to form inclusion bodies. It was recovered from the periplasm with 7 M guanidine hydrochloride and renaturing in 10 mM Tris-HCl, 1 mM EDTA, 300 mM arginine, pH 6.5. Proteins were further purified using successive rounds of ion exchange chromatography.

During purification of some the RAP-containing proteins, an affinity chromatography step with heparin-Sepharose (Pharmacia) was also included.

Tissue Culture—Murine L-929 cells, green monkey COS cells, and human A431 cells were obtained from ATCC. Chinese hamster ovary line, CL6, and mutants 221-1 and 13-51 were from Dr. S. Leppa (NIH). All lines were maintained in RPMI 1640 medium, 5% fetal bovine serum with penicillin and streptomycin. 51Cr cells, derived from Spo-doptera frugiperda pupal ovarian tissue were from Dr. J. T. Schiller (NIH). SL2 cells, derived from Drosophila melanogaster, were from Dr. I. Krasnoselskaja (NIH). All lines were maintained in Bac-V medium from Stratagene supplemented with 5% fetal bovine serum.

Cytotoxicity Assay—Cells at 10^4 per well (24-well plates) were seeded 1 day prior to evaluating the cytotoxicity of the toxin-related proteins. Various concentrations of toxins and ligand-toxins were added to cells for 20 h at 37°C. At the end of this period, [³H]leucine was added to a final specific activity of 2 μCi/ml for a further 1 h. To remove unincorporated radioactivity, monolayers were washed with phosphate-buffered saline. Proteins were then precipitated with trichloroacetic acid and the radioactivity per well determined by solubilizing the monolayers with 0.1 N NaOH and counting in a liquid scintillation counter.

The results are expressed as percentage of control wells to which no toxin was added.

To determine the effect of adenovirus on cytotoxicity of RAP-PE, 96-well plates were used. Cells at 4 x 10^4 per well (96-well plates) were seeded 1 day prior to evaluating the cytotoxicity. Various concentrations of toxins and RAP-PE were added to cells for 2 h with or without non-toxic amounts of adenovirus type 2 (approximately 3 μg/ml) at 37°C. At the end of this period, the existing medium was substituted by fresh medium containing [³H]leucine (0.5 μCi/ml) and cells were incubated further at 37°C for another 1 h.

Competition Assay—PE at 300 ng/ml, in the presence or absence of potential competitors, was added to cells for 4 h at 37°C. During the last hour of the 4-h incubation, [³H]leucine was added to a final specific activity of 2 μCi/ml. To determine the extent of inhibition of protein synthesis, the monolayers were processed as described above.

Receptor Binding Studies—LRP from bovine liver was affinity purified on a DT-RAP column, as described (32). Different amounts of purified receptor were applied to individual wells of an Immuno microtiter plate. Following the addition of bovine serum albumin to block nonspecific binding, PE or DT-RAP with or without competitor were added to the receptor-coated wells. Evidence of PE and DT-RAP binding was obtained by adding peroxidase-labeled (J ackson Laboratories) affinity-purified rabbit anti-PE or peroxidase-labeled affinity-purified horse anti-DT.

RESULTS

Construction and Expression of RAP-toxin Gene Fusions—A polymerase chain reaction fragment encoding full-length RAP was generated from pRAP1-23. Subsequently, RAP-PE and DT-RAP gene fusions were constructed, as illustrated in Figs. 1 and 2. In the RAP-PE construct (pET39PA2), RAP replaced amino acids 1-103 at the N terminus of PE. For the generation of DT-RAP (pVCDT39-1), RAP was positioned on the C-terminal side of amino acids 1-388 of DT. Expression levels in E. coli of both hybrid toxins ranged from 20 to 30% of the total cell protein.

The DT-RAP fusion protein accumulated in the periplasm, while the RAP-PE hybrid was found mostly in the cell cytoplasm as a soluble protein. Since the DT-RAP fusion lacked a signal sequence, the observed secretion to the periplasm had not been expected.

Properties of RAP-PE and DT-RAP—To determine if the two RAP-toxin fusions retained binding activity, a series of binding and ligand blocking experiments were devised. Since native RAP binds heparin, we first assessed the heparin-binding ca-
Pacity of both RAP-PE and DT-RAP. Both hybrid proteins bound to a heparin-Sepharose column and both were eluted with the application of a linear gradient of sodium chloride: RAP-toxins eluted between 0.8 and 1.0 M NaCl (data not shown). Since similar conditions are used to elute native RAP, we concluded that the heparin binding activity of RAP was unaffected by a fusion to either the N terminus of PE or the C terminus of DT.

Next we investigated whether or not the RAP fusions retained their ability to interact with LRP. Results obtained with an enzyme-linked immunosorbent assay indicated that RAP-PE bound to LRP at both pH 5.5 and 7.2, and that the addition of heparin (9 μg/ml) as a competitor reduced this binding to very low levels (Fig. 3). Previously, Moestrup and Gliemann (13) reported that the addition of heparin completely abolished the binding of RAP to LRP. Furthermore, when we preincubated heparin with heparinase its blocking activity was completely lost, indicating that specific sequences of monosaccharides within heparin were responsible for the inhibition. Using the same assay conditions, PE was shown to bind to LRP but with a much lower apparent affinity (Fig. 3). PE binding was not inhibited by heparin. In ligand blots following SDS-PAGE run under non-reducing conditions, DT-RAP was seen to interact with the heavy chain of LRP (data not shown). This binding was abolished when LRP was treated with β-mercaptoethanol. As a further indication that DT-RAP retained LRP binding activity, we report that immobilized DT-RAP could be used to affinity purify LRP from beef liver (data not presented).

**Fig. 1. Construction of plasmids encoding RAP-PE.** Boxes show the relative positions of coding sequences and regulatory elements: P lac and lacZ α, promoter and LacZ α encoding sequence; α10 and Tf α, fragments of T7 bacteriophage DNA which encode the α10 promoter and transcription termination signal, respectively; eta α10, sequences encoding a truncated version of PE that lacks the first 103 N-terminal amino acid residues and a fragment of α10MR/LRP, respectively; spa α and rap, sequences encoding the immunoglobulin-binding region of S. aureus protein A and RAP, respectively.

**Fig. 2. Construction of plasmids encoding DT-RAP and DT-PAI-1.** tox A, tox B*, and tox B-, sequences encoding fragment A and portions of DT B-fragment; pai-1, sequence encoding PAI-1. The remaining symbols are the same as in Fig. 1.
was seen when DT-RAP was added in the range of 11–33 nM. Activity of PE for L929 cells. Significant reversal of PE toxicity Fig. 4 shows that the addition of DT-RAP inhibited cytotoxicity of these cell lines. TGF-α was found in cells of Caenorhabditis elegans (33). These data suggest a wide distribution of this receptor in animals. To determine the cytotoxicity of RAP-PE and DT-RAP, relative to native PE, RAP-toxins were added to cell lines from five different species. These included: murine L929 cells, a line which is very sensitive to PE; COS cells, a green monkey kidney line, which is very sensitive to DT; A431, a human epithelial cell line; Chinese hamster ovary cells; and Sf9 insect cells. Because the RAP-toxins bound to LRP with higher affinity than native PE, it was expected that they might exhibit greater toxicity than PE itself. Based on previous results, this could be expected in six different epidermal growth factor receptor-positive cell lines, TGF-α PE40 was generally 10-fold more toxic than native PE (34). Surprisingly, the reverse was seen with the RAP-toxins. In all lines tested, PE was more active than either RAP-PE or DT-RAP (Table 1). In the two lines most sensitive to PE, L929 and COS, native PE was 80–500-fold more active than the hybrid toxins. These differences were present but less pronounced in the other three lines.

To determine if RAP toxin was being internalized to the endosomal compartment, cells were co-incubated for 2 h with RAP-PE and non-toxic amounts of adenovirus type 2 (approximately 1 μg/ml of virus). Previously, we showed that adenovirus disrupts endosomal membranes and releases the contents to the cytosol (35). Results indicated that when adenovirus and RAP-PE were added to cells together, cytotoxicity was enhanced by approximately 5-10-fold (Fig. 6). From this we conclude that RAP-PE binds and enters cells but is less toxic than the native toxin because the toxin portion of the hybrid is transported to a location which does not allow efficient translocation to the cytosol.

To examine possible reasons for the relatively low toxicity of the RAP-toxins, additional experiments were performed on three cell lines all having the same genetic background. Wild type (WT) Chinese hamster ovary cells (clone CL6) and two lines characterized as PE-resistant (13-5-1) and PE-supersensitive (221-1) were used. The 13-5-1 cells have no detectable LRP (16) while the 221-1 line appears to express slightly higher levels of LRP than wild type. Compared to WT, the LRP-
negative line was 100-fold resistant to PE, while the 221-1 was 3-fold more sensitive (Fig. 7). On WT and 221-1 cells RAP-PE was 5- and 10-fold less active than native PE, respectively (Fig. 7). While 13-5-1 cells were 10-fold resistant to RAP-PE compared to WT, the absolute activity of RAP-PE and PE for this cell line was quite similar. DT-RAP had about the same activity as RAP-PE on WT and 221-1 cells (Fig. 7). However, the 13-5-1 cells exhibited only slight resistance to DT-RAP. Thus, in the context of a RAP fusion, PE toxicity was much more dependent on delivery by LRP than was DT. Since DT can translocate from the endosomal compartment and PE must reach the endoplasmic reticulum, it is possible that internalization on any receptor will allow efficient delivery of DT to the cytosol but not PE (see "Discussion").

Construction and Cytotoxicity of RAP-PE Hybrids Composed of N-terminal and C-terminal RAP Fragments—To study the relationship between binding to LRP and cytotoxicity, we decided to take advantage of recently published data reporting that the N- and C-terminal portions of RAP can bind independently but less efficiently than full-length RAP to LRP and to the protein from the same family: gp330 (10–12). Therefore by replacing a small NotI-KpnI fragment in the plasmid pET39PA2 with NotI-KpnI fragments amplified from plasmid pET39-5 using either rap-NotI and RAP-KpnI or RAP-HindIII and rap-KpnI (see "Materials and Methods") primers we constructed plasmids pETN39PA5 and pET39CPA11. These plasmids encode RAP-PE hybrids that are composed of the N- and C-terminal portions of RAP, respectively. When the cytotoxic activities of these proteins were analyzed, the RAP fragment-toxin hybrids were found to be 5-10-fold more toxic than whole RAP-PE (Fig. 8).

Construction and Expression of a DT-PAI-1 Gene Fusion—To study the fate of another ligand that binds LRP, we constructed a fusion between DT and the protease inhibitor PAI-1 (Fig. 2).

Polymerase chain reaction was used to amplify the appropriate PAI-1 sequence (36) from total human cDNA. Primers were designed to produce a PAI-1 construct that lacked a signal sequence. The resulting plasmid called pVCDT-PAI-1-4 was transformed into BL21(DE3) cells and the hybrid protein (here called DT-PAI-1) was expressed. The expression level reached approximately 20% of the total cell protein. DT-PAI-1 had the expected molecular mass of 97.5 kDa and was recognized by horse antibodies to DT. Because it was insoluble, DT-PAI-1 was recovered from inclusion bodies by denaturation with guanidine hydrochloride and renatured at either pH 6.5
or 8.0. Although similar yields of soluble protein were achieved at either pH, the DT-PAI-1 renatured at the higher pH interacted with tPA less well than the hybrid renatured at pH 6.5. PAI-1 can exist in either an active or a latent (inactive) form (37). Over time, the active form converts into the latent form. Apparently, this conversion proceeds faster at pH 8.0 than at 6.5 (38).

Interaction of DT-PAI-1 with tPA—The interaction of PAI-1 with tPA produces a complex which is stable to treatment with SDS. To determine if DT-PAI-1 could interact with tPA in a similar fashion, the two were mixed and the formation of a stable complex analyzed by SDS-PAGE. Results indicated that a novel molecule of molecular mass 160 kDa was visualized (Fig. 9). In Western blot analysis, this complex reacted with antibodies to DT. To determine if DT-PAI-1 exhibited protease inhibitory activity, it was added to a reaction mixture comprised of tPA and the substrate N-methylsulfonyl-D-Phe-Gly-Arg-4-nitroanilide acetate. In the presence of DT-PAI-1, there was dramatic inhibition of substrate hydrolysis (data not presented).

Cytotoxicity of DT-PAI-1-tPA Complex—Once formed, DT-PAI-1-tPA should bind to the heavy chain of LRP. To determine if this complex was toxic for cells, the complex or DT-PAI-1 alone was added to either COS or A431 cells. There was no toxicity for A431 cells (data not shown). However, the complex was toxic for COS cells (Fig. 10). DT-PAI-1-tPA, which had low specific activity compared to either PE or DT, was about 16 times more toxic for COS cells than DT-PAI-1 alone.

DISCUSSION

PE enters cells by receptor-mediated endocytosis. Endocytosis is the beginning of the toxin pathway that results in the generation of an enzymatically active fragment, the translocation of this fragment to the cell cytosol and, ultimately, the inhibition of protein synthesis. Previously, we reported that PE binds to the heavy chain of LRP and most likely uses this receptor for endocytic uptake (14). LRP is a multiligand receptor whose function is the clearance and degradation of proteases and ligands related to lipid metabolism. Therefore, most of LRP ligands end up in the lysosome where they are degraded. For PE, some percentage of entering molecules some-

Fig. 8. Cytotoxicity of PE, RAP-PE, and RAP fragments PE for COS cells. Proteins were added to COS cells for an overnight incubation at 37 °C. At the end of the incubation period the level of protein synthesis was determined by measuring the incorporation of [3H]leucine into cellular protein. ■, RAP (1–323)-PE; ○, RAP (1–175)-PE; Δ, RAP (172–323)-PE; +, PE. The numbers in parentheses indicate the residues from RAP that were present in each construction.

Fig. 9. Interaction of DT-PAI-1 with tPA. The ability of DT-PAI-1 to interact with tPA was assessed by co-incubation of the two proteins followed by analysis on SDS-PAGE and staining with Coomassie Blue. Lanes: 1, DT-PAI-1 incubated with tPA; 2, DT-PAI-1; 3, tPA; and 4, molecular weight markers.

Fig. 10. Cytotoxicity of DT-PAI-1-tPA and DT-PAI-1 for COS cells. Proteins were added to COS cells for an overnight incubation at 37 °C. At the end of the incubation period the level of protein synthesis was determined by measuring the incorporation of [3H]leucine into cellular protein. ■, PE; □, DT; ●, DT-PAI-1-tPA; Δ, DT-PAI-1.

how avoid this fate. Existence of an endoplasmic retention-like sequence at the C terminus of PE and results of mutational analysis of this sequence suggest that PE or its enzymatically active fragment is transported to the ER where it translocates to the cytosol.

Among the ligands that bind LRP, are RAP and the complex between tPA and PAI-1. To compare the fate of these ligands with PE, we constructed PE and DT hybrid proteins that have RAP or PAI-1 in place of the toxins’ receptor-binding domains. We found that DT-PAI-1 was able to inhibit the catalytic activity of tPA and form an SDS-resistant complex with this protease. This data suggest that the N terminus of PAI-1 is not essential for its interaction with tPA. Toxicity was clearly specific for the DT-PAI-1-tPA complex since the addition of DT-PAI-1 alone was at least 10-fold less active. However, compared with DT and PE, the DT-PAI-1 complexed with tPA exhibited a much lower level of toxicity for COS cells.

RAP-PE and DT-RAP were also less toxic than PE for mam-
ovarian and insect cells. We showed that these proteins retained their binding activity for the heavy chain of LRP. Since the RAP cDNA is fused at the 5’ end of the PE gene and 3’ end of the DT gene, it appears that neither the N or C terminus of RAP are required for interaction with LRP.

Among various cell lines we did not find a correlation between PE and RAP-toxin sensitivities. Nevertheless, we were able to see such a correlation when the toxins were tested on isogenic cell lines. In particular, a PE-resistant line, 13-5-1, that lacks detectable LRP protein was less sensitive to RAP-toxins. A second mutant, 221-1, that expressed higher amounts of LRP than wild type cells and was supersensitive to PE by 3-5-fold, was also more sensitive to RAP-toxins. The lack of a good correlation between PE and RAP-toxin toxicities may be explained by results of several authors who have demonstrated the existence of additional receptors for RAP besides LRP.

The affinity allows native PE to dissociate from LRP and thus to enhance toxicity by releasing more toxin than was possible in the absence of virus. Toxicity was enhanced by 5-10-fold.

In conclusion, we speculate that a relatively low binding affinity allows native PE to dissociate from LRP and thus to avoid the fate of other LRP ligands, which is to be degraded in the lysosome. Our data showing increased cytotoxicity when RAP fragments were fused to PE instead of whole RAP seems to support this hypothesis. This finding may have wide reaching implications for the design of recombinant immunotoxins.

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