Increased Cytotoxicity of Interleukin 2–Pseudomonas Exotoxin (IL2–PE) Chimeric Proteins Containing a Targeting Signal for Lysosomal Membranes†

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ABSTRACT: IL2–PE40 is a chimeric protein composed of human interleukin 2 (IL2) genetically fused to the amino terminus of a truncated form of pseudomonas exotoxin lacking its cell recognition domain (PE40). IL2–PE40 is extremely cytotoxic to IL2 receptor positive cells. This chimeric protein was found to be an effective and selective immunosuppressive agent for IL2 receptor targeted therapy in many models of disorders of the immune response where activated T-cells play a crucial role. In an attempt to produce an improved IL2–PE40 chimeric protein, we constructed new IL2–PE derivatives. This was done by inserting defined DNA sequences within the chimeric gene encoding IL2–PE40. Inserted sequences represent motifs of other proteins known to be targeted and/or sorted to specific compartments inside or outside the cell. One of the proteins, IL2–PE40(LAP+DUP), containing a targeted signal for lysosomal membrane, was 2–3-fold more active than IL2–PE40. The insertion of the LAP sequence also increased the cytotoxicity of another IL2–PE derivative, IL2–PE66^{4Glu}. Our results suggest that a selective targeting of IL2–PE chimeric proteins to lysosomes may enable the proteins to reach the cytosol more efficiently, thus improving its specific cytotoxicity. The LAP (lysosomal alkaline phosphatase) sequence may be used as a common motif for increasing the cytotoxicity of other chimeric proteins to be used for targeted immunotherapy.

Chimeric cytotoxins are a novel class of targeted molecules designed to recognize and specifically destroy cells over-expressing specific receptors. These include cells involved in many disorders of the immune response. IL2–PE40 is a chimeric protein composed of human interleukin 2 (IL2)[1] genetically fused to the amino terminus of a modified form of pseudomonas exotoxin (PE) lacking its cell recognition domain (PE40) (Lorberboum-Galski et al., 1988a). Once it accesses the cytoplasm, PE irreversibly inhibits protein synthesis by its ADP ribosylation activity and causes cell death (Middlebrook & Dorland, 1984). IL2–PE40 is extremely cytotoxic to IL2 receptor positive cells (Lorberboum-Galski et al., 1988a,b). IL2–PE40 was found to be an effective and selective immunosuppressive agent for IL2 receptor targeted therapy in many models of disorders of the immune response where activated T-cells play a crucial role.

Using a highly purified preparation of the chimeric protein (Bailon et al., 1988), IL2–PE40 was shown to (a) delay and mitigate adjuvant-induced arthritis in rats (Case et al., 1991); (b) significantly prolong the survival of vascularized heart allografts in mice (Lorberboum-Galski et al., 1989); (c) reduce the incidence and severity of experimental autoimmune uveoretinitis (EAU) in rats (Roberge et al., 1989); (d) suppress the growth of a T-cell lymphoma in mice (Kozak et al., 1990); (e) prevent the characteristic features of experimental autoimmune encephalomyelitis (EAE) in rats and mice (Beraud et al., 1991; Rose et al., 1991); and (f) significantly reduce the clinical rejection score and cumulative rejection rate in orthotropic corneal grafts in rats (Herbort et al., 1991).

A chimeric protein such as IL2–PE40, given as a potential immunosuppressive reagent, traverses a long path until it reaches the cytosol, where its substrate, EF-2, is situated. The chimeric protein needs to bind to a specific receptor on the cell surface of the target cell, clusters into coated pits, and is delivered into an intracellular acid compartment—the receptosome. These vesicles move away from the cell surface by saltatory motion and fuse with the Golgi. As a result of a sorting process that occurs in the Golgi, much of the toxin is sent to the lysosome to be degraded, some is released into the extracellular fluid, and a small amount escapes into the cytosol where it acts on its substrate. Thus, the specific cytotoxicity of a chimeric protein is dependent upon the successful combined operation of those sequential steps.

Ongoing studies indicate that the translocation of PE, and most probably of IL2–PE40, is the rate-limiting step in its cytotoxicity. Only a low percentage of the molecules enters the cytosol to stop their protein synthesis. Today, increasing the translocation efficiency of a chimeric protein is one of the major goals in developing molecules for selective and specific killing of target cells.

In attempting to produce an improved IL2–PE chimeric protein, we introduced a few DNA sequences along the chimeric gene encoding IL2–PE40. Selected sequences represented various signals known to be involved in targeting and sorting different proteins into specific compartments inside or outside the cell; targeting included passage of proteins through intracellular membranes.

One of the new chimeric proteins constructed, IL2–PE40-(LAP+DUP), containing a targeting signal for lysosomal membrane, was 2–3-fold more active than IL2–PE40. Our results suggest that targeting the IL2–PE chimeric proteins directly to lysosome, by inserting a defined sequence for the lysosomal membrane, may allow a higher percentage of the chimeric proteins to reach the cytosol, thus improving its cytotoxic activity. IL2–PE(LAP+DUP) may be used as potent immunosuppressive agents for targeted immunotherapy.

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[1] Abbreviations: IL2, interleukin 2; PE, pseudomonas exotoxin; LAP, lysosomal alkaline phosphatase; DUP, duplication.

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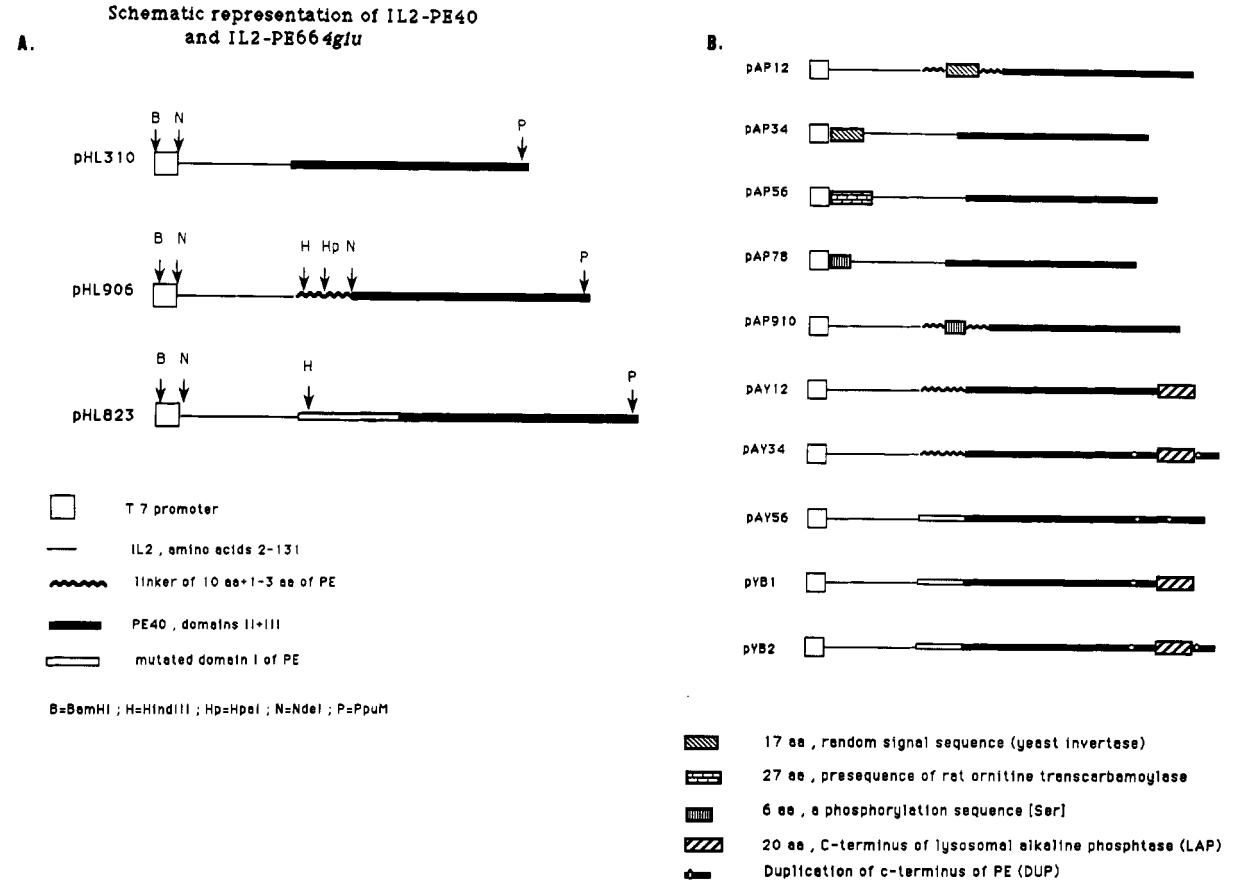

FIGURE 1: Schematic representations of IL2–PE40, IL2–PE66^{4Glu} (A), and newly designed IL2–PE chimeric proteins (B). pHL310 and pHL906 are encoding IL2–PE40. pHL823 encodes IL2–PE66^{4Glu}.

This sequence may serve as a general motif for increasing the cytotoxicity of various other chimeric proteins.

MATERIALS AND METHODS

Reagents. Restriction endonucleases and T4 DNA ligase were purchased from Boehringer Mannheim and used under conditions recommended by the supplier. [^{3}H]Leucine (48 Ci/mmol) and [^{3}H]thymidine (55 Ci/mmol) were purchased from Amersham Corp. Human IL2 was a gift from Hoffmann-La Roche.

Bacterial Strains, Plasmids, and Oligonucleotide Synthesis. *Escherichia coli* strain HB101 was used for the transformation and preparation of plasmids. BL21 (λDE3), which carries a T7 RNA polymerase gene in a lysogenic and inducible form (Studier & Moffatt, 1986), was used as the host for the synthesis of the chimeric proteins. pHL310, which encodes IL2–PE40, was described previously (Lorberboum-Galski et al., 1988a). In this plasmid, the two moieties of the chimera, encoding IL2 and PE40, are closely located with only one amino acid (Ile) separating them. In plasmid pHL906, these two parts are separated by a 10 amino acid synthetic linker. This synthetic linker contains a few unique restriction sites (Figure 1). IL2–PE40 encoded by these two plasmids has identical cytotoxic activity (our unpublished observations). Synthetic oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified on oligonucleotide purification cartridges.

Protein Synthesis Inhibition Assay. Cytotoxic activities of IL2–PE derivatives were evaluated by the inhibition of protein synthesis, as measured by the [^{3}H]leucine incorporation on HUT102 cells (Lorberboum-Galski et al., 1988a,b). Various concentrations of IL2–PE derivatives, diluted with 0.2% bovine serum albumin in phosphate-buffered saline, were added to 2×10^4/0.2 mL cells in 96-well plates for 20 h, followed by a 4–6-h pulse with 2 μCi of [^{3}H]leucine. Results are expressed as a percent of control experiments in which the cells were not exposed to any chimeric protein. All assays were carried out in four separate experiments in triplicate, and statistical comparisons were made using the student's *t*-test.

Cell Proliferation Inhibition on PHA Blasts. Human peripheral blood lymphocytes were separated using a Ficoll gradient (Ficoll Paque Pharmacia). Lymphocytes were cultured in 5% CO_2 in air in Dulbecco's modified medium supplemented with 5% heat-inactivated (56 °C, 30 min) fetal bovine serum, 200 μg/mL L-glutamate, 50 μg/mL penicillin, 50 μg/mL streptomycin, 50 μg/mL glutamine, and 5×10^{-5} M β-mercaptoethanol. PHA-P (Sigma L9132, 5 μg/mL) was added at the start of the culture. Cells were harvested after 3 days and used for assays (2×10^4 cells/200 μL/well). rIL2 (10 units/mL) was added to the culture after 3 days to maintain the growth and viability of the PHA blasts during the assay period. At the same time, different concentrations of chimeric proteins, diluted in phosphate-buffered saline and 0.2% bovine serum albumin, were added. After a 48-h incubation, 1 μCi of [^{3}H]thymidine was added to each well for 13–20 h of labeling. Cells were harvested on filters, and the incorporated radioactivity was measured (Lorberboum-Galski et al., 1990).

Gel Electrophoresis and Immunoblotting. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 10% gels as described by Laemmli (1970). The gels were stained with Coomassie blue or silver-staining reagent. For immunoblotting, electrophoresed samples were

transferred from the gels to nitrocellulose paper and immunoblotted as described (Lorberboum-Galski et al., 1988a), using antibodies to native PE or to human IL2. A Vector staining kit was used as recommended by the manufacturer. The antibody to native PE has been described (Hawng et al., 1987). Antibodies to human IL2 were a gift from Hoffmann-La Roche.

Protein and ADP-Ribosylation Assays. Protein concentration was measured by a Bradford protein assay kit (Bio-Rad) using BSA as the standard. The ADP-ribosylation activity of tested samples was measured using wheat germ extracts enriched in elongation factor 2, as described previously (Collier & Kandel, 1971).

Protein Expression and Partial Purification. The chimeric proteins were expressed in *E. coli* strain BL21 (λDE3). Induction was performed using isopropyl β-D-thiogalactoside (IPTG, 1 mM final concentration). A pellet of expressed cells was suspended in TE buffer (50 mM Tris (pH 8.0) and 1 mM EDTA), sonicated (six 30-s bursts, 100 W), and centrifuged at 10000*g* for 15 min. The supernatant (soluble fraction) was removed and kept for analysis. The pellet was denatured in 4 vol (v/w) of extraction buffer (7 M guanidine hydrochloride, 0.1 M Tris (pH 8.0), 1 mM EDTA, and 5 mM DTT) and sonicated (six 30-s bursts, 100 W). The suspension was stirred for 1 h at 4 °C. The suspension was cleared by centrifugation at 12000*g* for 15 min, discarding the pellet. The cleared supernatant was then diluted 4-fold with PBS buffer and dialyzed against PBS. The dialyzed material was centrifuged at 12000*g* for 10 min. The resulting supernatant (insoluble fraction, guanidine hydrochloride-treated) was used as a source for the chimeric proteins for most experiments.

Internalization of Chimeric Proteins into Target Cells. The internalization of chimeric proteins ($LYAY_{1,2}$, $LYAY_{3,4}$, $LYAY_{5,6}$, and IL2–PE40) into target cells was assayed by incubating the proteins (15 μg, total protein concentration of the insoluble fraction, guanidine hydrochloride-treated, of each protein) with HUT102 cells for 3 h. At the end of the incubation, cells were washed four times with phosphate buffer (PBS), saving samples from each washing step. Cells were then lysed with lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP40, 1% deoxycholic acid, and 0.1% SDS containing 1 mM PMSF) and incubated for 15 min at room temperature. The cell lysate was centrifuged at 9000*g* for 10 min. Pellet (dissolved in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA (TE buffer)) and supernatant fractions of the lysed cells were kept for analysis. Samples of the growing media, the lysed cells, and the washing steps were subjected to SDS–PAGE analysis. Electrophoresed samples were transferred from gels to nitrocellulose and immunoblotted using αPE antiserum.

Plasmid Construction. Defined DNA sequences were inserted into pHL310 and pHL906 encoding IL2–PE40 or into plasmid pHL823 encoding IL2–$PE66^{4Glu}$. Sequences inserted at the amino terminus of the chimeric protein were introduced into the *Nde*I site in plasmid pHL310 (plasmids $pAP_{1,2}$, $pAP_{5,6}$, and $pAP_{7,8}$) (Figure 1). Sequences introduced into the synthetic linker were inserted into *Hin*dIII/*Hpa*I sites within plasmid pHL906 (plasmids $pAP_{3,4}$, and $pAP_{9,10}$). The LAP sequence was inserted into a *Ppu*MI site located at the carboxy terminus of plasmid pHL906 or pHL823, obtaining plasmids $pAY_{1,2}$ and pYB_1. The LAP sequence was designed to contained a unique *Dra*III restriction site at its carboxy terminus. Thus, plasmids $pAY_{1,2}$ and pYB_1 were cut with *Dra*III to insert an oligonucleotide encoding the carboxy terminus of PE, as a duplication (plasmids $pAY_{3,4}$ and pYB_2, respectively). All new plasmids were characterized by restriction analysis and their sequences confirmed by DNA sequence analysis.

Table 1: Oligonucleotides Used for Inserting Defined DNA Sequences into IL2–PE40 Chimeric Genes

	oligonucleotides	inserted within plasmids
I.	5'ATG CTT TTT CCA CGT GCT TGG TGG CTT ATG CCA GTA ATC CCA GTA GAA ATG 3'	$pAP_{1,2}$; $pAP_{3,4}$
II.	5' ATG CTG TCT AAT TTG AGG ATC CTG CTC AAC AAG GCA GCT CTT AGA AAG GCT CAC ACT TCC ATG GTT CGA AAT TTT CGG TAT 3'	$pAP_{5,6}$
III.	5' TCT GCT ATC CGT CGT GCT TCT GTT GCT 3'	$pAP_{7,8}$; $pAP_{9,10}$
IV.	5' GAC CTG AAG CGT ATG CAG GCT CAG CCG CCG GGT TAC CGT CAC GTT GCT GAC GGT GAA GAC CAC GCT GTG TAG 3'	$pAP_{1,2}$; pB^{Y}_{1}
V.	5' CCC GGC AAA CCG CCG CGC GAG GAC CTG AAG TAA 3'	$pAY_{3,4}$; pB^{Y}_{2}

RESULTS

In our attempt to produce a more potent IL2–PE chimeric protein, we introduced a few defined DNA sequences into the chimeric gene encoding IL2–PE40. Selected sequences represent various signals known to be involved in targeting and/or sorting different proteins into specific compartments inside or outside the cell. Targeting includes the passage of proteins through intracellular membranes. Sequences (Table 1) include the following: (a) a signal sequence for secretion composed of a random sequence of 17 amino acids, which was obtained by digestion of genomic DNA from human peripheral blood lymphocytes, that successfully replaced the natural sequence of invertase from yeast (Kaiser et al., 1987); (b) a sorting signal for mitochondria, whose sequence represents the first 27 amino acids of the sorting signal of rat ornithine transcarbamylase, which is found in the mitochondrial matrix (Hartl et al., 1989); (c) a common sequence for serine phosphorylation, a known general target for serine phosphorylation that is recognized by a cAMP-dependent protein kinase (Kemp & Pearson, 1990) but is longer (9 amino acids) than the common XRRXSX sequence; and (d) a signal for internalization and lysosomal targeting, whose sequence is located at the cytoplasmic tail of a lysosomal protein, lysosomal alkaline phosphatase (LAP), and was found to be necessary and sufficient for targeting to lysosomes (Peters et al., 1990). Sequences (Table 1) had been introduced along the chimeric genes: at the NH_2 terminus, within the synthetic linker, and at the COOH terminus carried on two plasmids (Figure 1 and Table 2).

New Chimeric Cytotoxins—General Characteristics. Newly designed chimeric cytotoxins were expressed in *E. coli*. Proteins were characterized by gel electrophoresis (Figure 2) and Western blot analysis (Figure 3), using antibodies against PE (Figure 3A) and IL2 (Figure 3B). As seen in Figures 2 and 3, all new chimeras react with both antibodies, thus confirming the cloning and production of in-frame, full-length chimeric proteins.

A profile of whole cell extracts from cells expressing the different new proteins revealed that the expression levels of the new chimeric proteins are high: 2%–10% of total proteins (Figure 2A,B) and similar to that of the original IL2–PE40. The expression of only one protein, $EXAP_{3,4}$, encoded by plasmid $pAP_{3,4}$, is relatively low (Figure 2A, lanes 4 and 5; Figure 3, lane 2).

Table 2: New IL2–PE Chimeric Proteins

plasmid	protein	role and target of sequence	features of sequence	site of insertion into IL2–PE40	unique restriction site	length of sequence
pAP_{1-2}	$EXAP_{1-2}$	secretion outside the cell	hydrophobic, no charged amino acids	amino terminus	*Bst*XI	17aa
pAP_{3-4}	$EXAP_{3-4}$	secretion outside the cell	hydrophobic, no charged amino acids	between IL2 and PE40	*Bst*XI	17aa
pAP_{5-6}	$MTAP_{5-6}$	mitochondria	rich in positively charged amino acids, no acidic acid, folded into α-helix	amino terminus	*Bam*HI	27aa
pAP_{7-8}	$PHAP_{7-8}$	serine phosphorylation	site for serine phosphorylation	amino terminus		6aa
pAP_{9-10}	$PHAP_{9-10}$	serine phosphorylation	site for serine phosphorylation	between IL2 and PE40		6aa
pAY_{1-2}, pYB_1[a]	$LYAY_{1-2}$, YB_1	lysosome	site for tyrosine phosphorylation	carboxy terminus[b]	*Dra*III, *Bst*eIII	20aa
pAY_{3-4}, pYB_2[a]	$LYAY_{3-4}$, YB_2	lysosome plus duplication of PE COOH terminus		carboxy terminus of pAY_{1-2}[b]		10aa

[a] Plasmids pYB_1 and pYB_2 are encoding IL2–$PE66^{4Glu}$ derivatives. [b] Sequence inserted into IL2–$PE66^{4Glu}$.

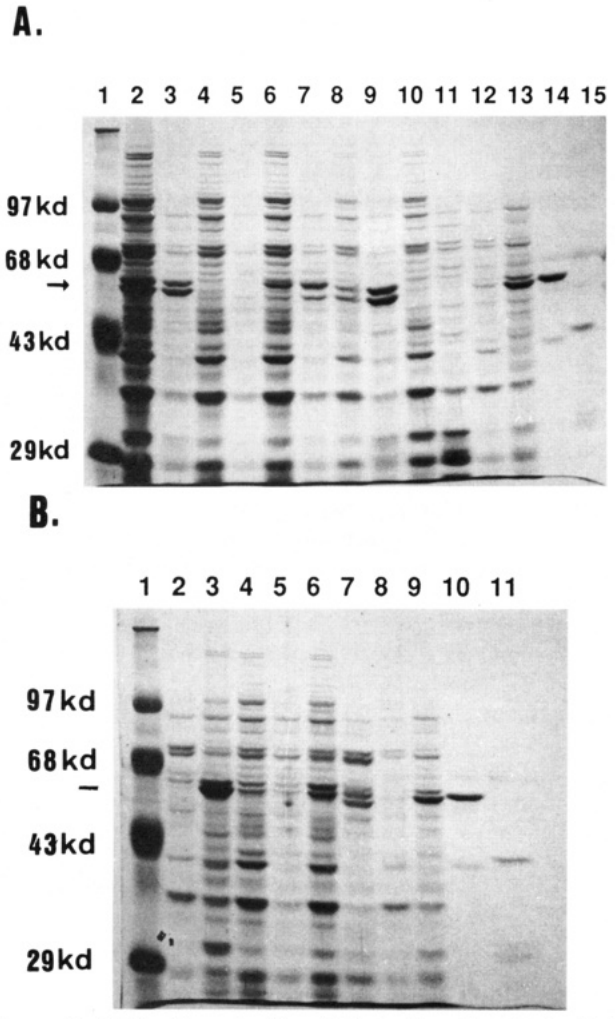

FIGURE 2: SDS–polyacrylamide gel electrophoresis analysis of cell fractions containing new IL2–PE chimeric proteins. Samples were mixed with Laemmli sample buffer (13) and boiled (100 °C) for 3 min. Samples of 10 mg (total protein concentration) were loaded onto an SDS–10% polyacrylamide gel. (A) Lane 1, markers; lane 2, $EXAP_{1-2}$ whole cell extract; lane 3, $EXAP_{1-2}$ insoluble fraction; lanes 4 and 5, cell fractions containing $EXAP_{3-4}$ (fractions are as in lanes 2 and 3); lanes 6 and 7, cell fractions containing $MTAP_{5-6}$ (fractions are as in lanes 2 and 3); lanes 8 and 9, cell fractions containing $PHAP_{7-8}$ (fractions are as in lanes 2 and 3); lanes 10 and 11, cell fractions containing $PHAP_{9-10}$ (fractions are as in lanes 2 and 3); lanes 12 and 13, cell fractions containing IL2–PE40 (encoded by pHL906; fractions are as in lanes 2 and 3); lanes 14 and 15, purified IL2–PE40 and PE40, respectively. (B) Lane 1, markers; lanes 2–9, cell fractions containing $LYAY_{1-2}$, $LYAY_{3-4}$, $LYAY_{5-6}$, and IL2–PE40 (encoded by pHL310), respectively. For each IL2–PE derivative, fractions are as in lanes 2 and 3 of $EXAP_{1-2}$. Lanes 10 and 11, purified IL2–PE40 and PE40, respectively. $LYAY_{5-6}$ is IL2–PE40(+DUP), encoded by pAY_{5-6}.

The new chimeric proteins have stabilities similar to that of IL2–PE40, except for $PHAP_{9,10}$. This protein encoding by $pAP_{9,10}$ is unstable: more than 50% of the expressed protein is degraded, as demonstrated by the immunoblot (Figure 3, lane 5). This degradation product is observed only when αPE antibodies are used, and it comigrates with PE40. Thus, insertion of a site for serine phosphorylation between the IL2 and PE40 moieties (in $PHAP_{9,10}$) most probably introduces a sensitive protease cleavage site, resulting in a degradation product that consists of only the PE portion.

Table 3: ADP-Ribosylation Activity of Newly Designed IL2–PE Chimeric Proteins

protein	% activity[c]	protein	% activity[c]
$EXAP_{1-2}$	85	$LYAY_{1-2}$[a]	113
$EXAP_{3-4}$	42	$LYAY_{3-4}$[b]	131
$MTAP_{5-6}$	84	YB_1[d]	125
$PHAP_{7-8}$	127	YB_2[d]	83
$PHAP_{9-10}$	15		

[a] $LYAY_{1,2}$ was termed IL2–PE40(LAP). [b] $LYAY_{3,4}$ was termed IL2–PE40(LAP+DUP). [c] Results are expressed as the percentage of activity from that of IL2–PE40 or [d] IL2–$PE66^{4Glu}$. In each example, identical protein amounts (total protein concentration) were tested.

All new proteins accumulate in both the soluble and insoluble fractions of expressing cells (results not shown). However, the insoluble fraction is the enriched fraction for the proteins, similar to the accumulation of IL2–PE40 expressed under identical conditions. Therefore, the introduction of various DNA sequences at different sites along the chimeric gene encoding IL2–PE40 had no effect on the subcellular accumulation of the various derivatives within the expressed cells. The insoluble fraction, guanidine hydrochloride-treated (see Materials and Methods), was used as a source of the new IL2–PE chimeric cytotoxins.

Enzymatic Activity of New Chimeric Cytotoxins. The enzymatic activity of the chimeric proteins is donated by the PE40 moiety of the molecule and is essential for its cytotoxicity. Therefore, the ADP-ribosylation activity was tested for the new proteins. Most of the new proteins exhibited enzymatic activity in vitro similar to that of IL2–PE40, except for $EXAP_{3,4}$ and $PHAP_{9,10}$ (Table 3). $EXAP_{3,4}$ exhibits enzymatic activity that is 42% of the activity of IL2–PE40. However, the expression levels of $EXAP_{3,4}$ are low (Figure 2, lane 4; Figure 3, lane 2). As enzymatic activity is tested in samples exhibiting identical protein amounts (total protein concentration) of the various chimeric cytotoxins, amounts of the chimera are low in this preparation compared to other preparations. Thus, $EXAP_{3,4}$, most probably, exhibits enzymatic activity similar to the other chimeras. $PHAP_{9,10}$ has very low ADP-ribosylation activity, demonstrating only 15% of the activity of IL2–PE40. As seen in Figure 2 (lanes 10 and 11) and Figure 3 (lane 5), $PHAP_{9,10}$ is mostly degraded in the expressing cells. However, degradation products react with αPE (Figure 3A, lane 5) and are expected to exhibit normal enzymatic activity.

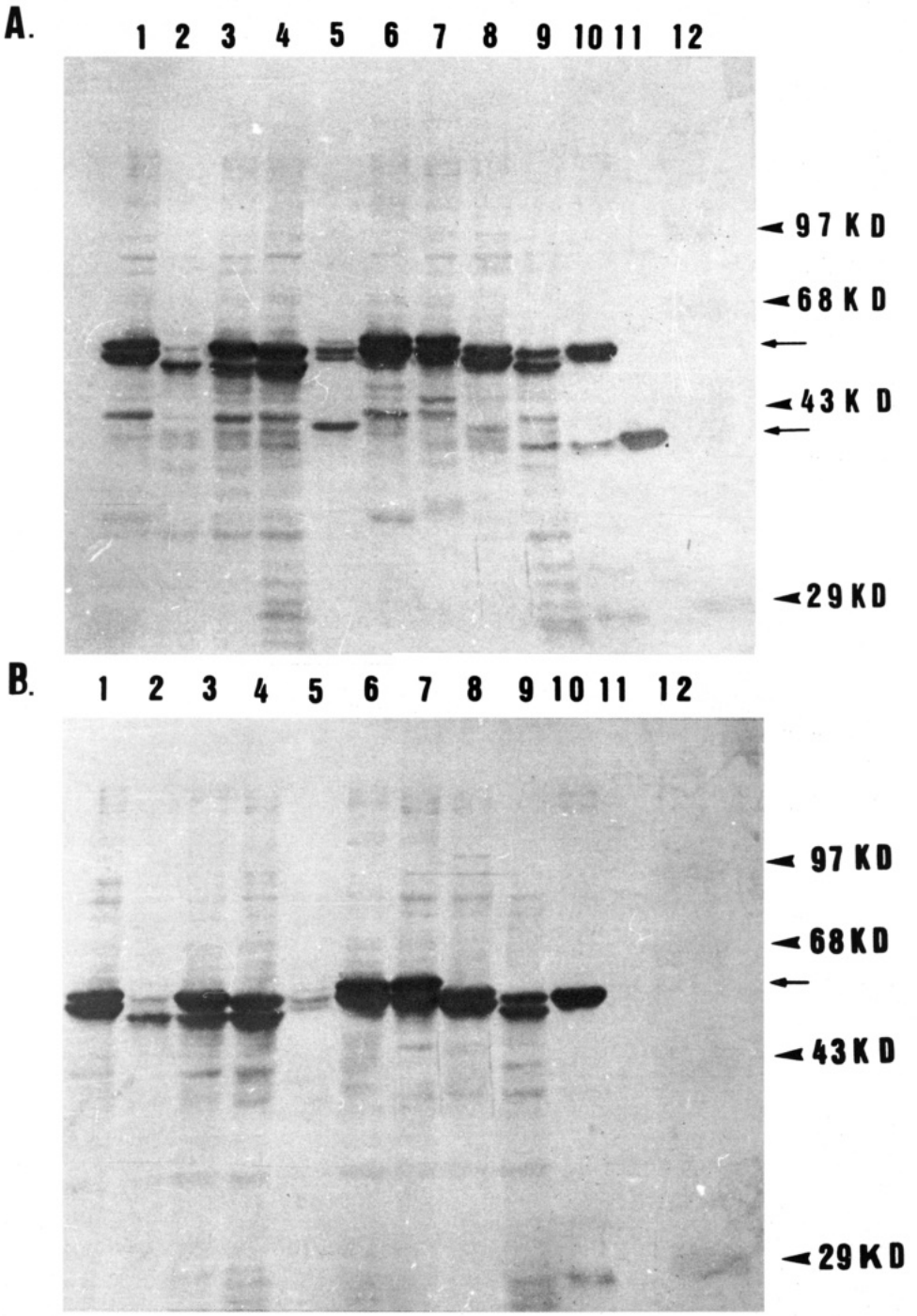

FIGURE 3: Immunoblotting of insoluble fractions containing various IL2–PE derivatives with antibodies to PE (A) and IL2 (B). Samples of 10 μg of protein (total protein concentration) were loaded onto a SDS–10% polyacrylamide gel. Electrophoresed samples were transferred from the gels to nitrocellulose and processed as described in the Materials and Methods section. (A) Lanes 1–7, insoluble fractions containing $EXAP_{1-2}$, $EXAP_{3-4}$, $MTAP_{5-6}$, $PHAP_{7-8}$, $PHAP_{9-10}$, $LYAY_{1-2}$, and $LYAY_{3-4}$; lanes 8 and 9, IL2–PE40 encoded by pHL310 and pHL906, respectively. Lanes 10 and 11, purified IL2–PE40 and PE40, respectively. Lane 12, markers. Blotting was performed using anti-PE antibodies. Upper arrow indicates IL2–PE chimeric proteins; lower arrow indicates PE40. (B) αIL2: Lanes are as described for PE (A).

Cytotoxicity of New Chimeric Cytotoxins on HUT102 Cells. New chimeric cytotoxins were tested on HUT102 cells, which are human T-cells expressing high levels of the high-affinity IL2 receptors. By comparing the amounts of chimeric protein causing 50% inhibition of protein synthesis (ID_{50} values), only one protein, $LYA_{3,4}$ [termed IL2–PE40-(LAP+DUP)], was more active than the original IL2–PE40. This protein, containing a targeting signal for the lysosome membrane, exhibited a 2–3-fold increase in its specific activity (Figure 4 and Table 4). The increase in cytotoxicity was observed repeatedly in all experiments.

Protein $LYA_{1,2}$ [IL2–PE40(LAP)], containing only the LAP sequence at the COOH terminus of the chimera, had no cytotoxic activity on HUT102 cells (Figure 4). However, it was reported that blocking the COOH terminus of native PE causes complete loss of its biological activity (Chaudhary et al., 1990). As seen in Figure 4, the LAP sequence was unable to restore the activity of the blocked PE. It was reported that a modified carboxy sequence of PE (KDEL, instead of the authentic sequence REDLK) repeated three times [$(KDEL)_3$] is more active than native PE ending in REDLK (Seetharam et al., 1991). Therefore, in order to establish that the increased

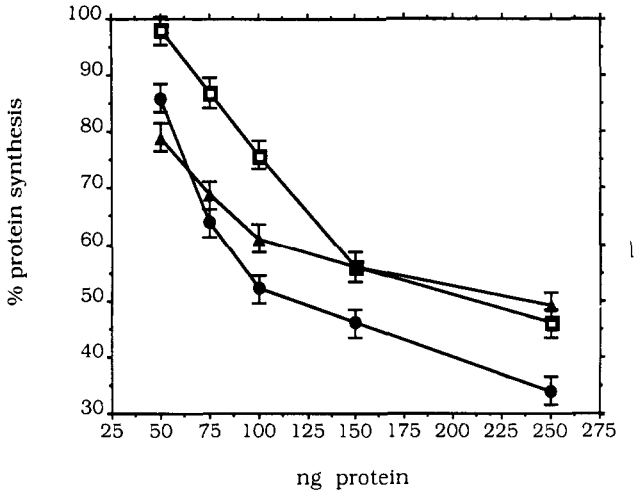

FIGURE 4: Cytotoxic activity of IL2–PE40(LAP+DUP) on HUT102 cells: □, IL2–PE40; ▲, IL2–PE40(DUP); and ●, IL2–PE40-(LAP+DUP). Insoluble fractions containing the IL2–PE chimeric proteins were added at various concentrations to the cells (according to total protein concentrations). [^{3}H]Leucine incorporation into cellular protein was measured as described in the Materials and Methods section. Results are expressed as the percent of control cells not exposed to any chimeric protein. Error bars are SE.

Table 4: Cytotoxicity of IL2–PE Derivatives[a]

chimeric protein	ID_{50}, ng (total protein concentration)	
	HUT102 cells	human activated lymphocytes
IL2–PE40(LAP+DUP)	125 ± 4	*b*
IL2–PE40(DUP)	250 ± 8	*b*
IL2–PE40	231 ± 7	*b*
IL2–PE66^{4Glu}(LAP+DUP)	58 ± 2	220 ± 6
IL2–PE66^{4Glu}	152 ± 3	708 ± 21

[a] The values are the mean of four separate experiments (performed with different expressions) done in triplicate ± SE. ID_{50} is the concentration of a chimeric protein (total protein concentration of the insoluble fraction) that inhibits protein synthesis by 50% compared to control cells not exposed to any chimeric protein. [b] Assays performed with the given concentrations (see Figure 5) did not reach the ID_{50} values.

cytotoxicity of IL2–PE40(LAP+DUP) is due to the inserted LAP sequence and not due to the duplication of the PE terminus, we constructed an additional IL2–PE derivative, in which we duplicated only the original carboxy terminus of PE–REDLK, omitting the LAP sequence. This chimeric protein, IL2–PE40(DUP) (encoded by $pAY_{5,6}$; Figure 2B, lanes 6 and 7), has a cytotoxic activity similar to that of IL2–PE40 when tested on HUT102 cells (Figure 4 and Table 4). Thus, the increased cytotoxic activity of IL2–PE40-(LAP+DUP) is contributed by the insertion of the LAP sequence.

As the LAP sequence is also known to accelerate the internalization of the molecules that contain it (Peters et al., 1990), the increased cytotoxicity of IL2–PE40(LAP+DUP) could be due to the increased efficiency of internalization of this new chimera. To study this question, proteins IL2–PE40-(LAP), IL2–PE40(DUP), IL2–PE40(LAP+DUP), and the authentic IL2–PE40 were incubated for 3 h at equally high concentrations (15 μg of the insoluble fraction of each chimeric protein) with HUT102 cells. We then compared the amounts of the different proteins within the cells (and growing media), following incubation, by immunoblot analysis using αPE antibodies (Ogata et al., 1990). IL2–PE40(LAP+DUP) and the authentic IL2–PE40, as well as the other IL2–PE40 derivatives, could be detected within lysates of the cells at similar levels (results not shown). Thus, the possibility that

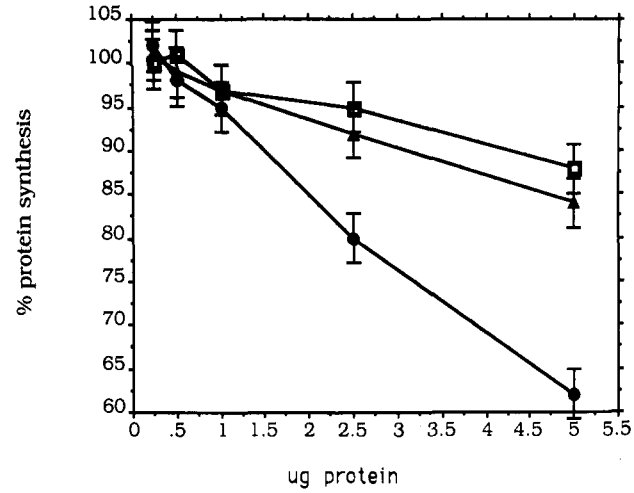

FIGURE 5: Inhibition of DNA synthesis of human PHA blasts by IL2–PE40(LAP+DUP). Lymphocytes (1×10^6 mL) from human donors were stimulated with PHA-P (5 μg/mL) for 3 days. The cells were then washed and incubated in the culture medium containing rIL2 (10 units/mL) in the absence or presence of IL2–PE40-(LAP+DUP) (●), IL2–PE40(DUP) (▲), and IL2–PE40 (□). After 40–48 h of incubation, [^{3}H]thymidine incorporation was measured. Results are expressed as the percent of control, activated lymphocytes not exposed to chimeric proteins. The data shown represent one out of four experiments performed with different donors. Error bars are SE.

the increased cytotoxicity of IL2–PE40(LAP+DUP) is due to the increased efficiency of its internalization into target cells is not likely.

IL2–PE40(LAP+DUP) was also tested on nontarget cells such as OVCAR 3 cells and showed no cytotoxicity (results not shown). All other new chimeric proteins constructed either had cytotoxic activities similar to IL2–PE40 or completely lost their cytotoxicity (Tables 4 and 5).

Effect of New IL2–PE Chimeric Cytotoxins on Human PHA-Activated Lymphocytes. Next, we tested whether IL2–PE40(LAP+DUP) is also cytotoxic to activated human T lymphocytes. We reported before that IL2–PE40 had poor cytotoxic activity against human-activated T lymphocytes (Lorberboum-Galski et al., 1990). Human peripheral blood lymphocytes were prepared and stimulated to grow by the addition of the mitogen PHA. These mitogen-stimulated cells (PHA blasts) express functional IL2 receptors on the cell surface and require IL2 for growth in vivo (Yamamoto et al., 1985). To test for cytotoxicity, IL2–PE40(LAP+DUP) and other IL2–PE derivatives were added for 48 h to lymphocytes that had been activated for 3 days, and then [^{3}H]thymidine incorporation was tested. As shown in Figure 5 and Table 4, mitogen-activated human lymphocytes are sensitive to IL2–PE40(LAP+DUP)-mediated cytotoxicity in a dose-dependent manner. Cytotoxicity is observed at a high concentration of the chimeric proteins. However, equivalent amounts of control proteins (a nonrelevant chimeric protein and PE40) added under the same conditions had no effect on DNA synthesis (results not shown). IL2–PE40(LAP+DUP) is about 2–3-fold more active than the original IL2–PE40 chimeric proteins or IL2–PE40(DUP). Thus, the increasing cytotoxicity of IL2–PE40(LAP+DUP) can be attributed to the insertion of the LAP sequence into IL2–PE40, rather than the duplication of the C-terminus of the molecule.

IL2–PE40 or IL2–PE40(DUP) barely have cytotoxic activity toward human activated T lymphocytes (Figure 5, Table 4). Insertion of the LAP sequence produced a molecule that showed some low activity on freshly activated human T cells.

Table 5: Characteristics of New IL2–PE Chimeric Proteins

protein	encoded by plasmid	level of expression	stability[b]	ADP-ribosylation activity	cytotoxic activity on HUT102 cells	cytotoxic activity on human activated lymphocytes
$EXAP_{1-2}$	pAP_{1-2}	high	stable	+++		
$EXAP_{3-4}$	pAP_{3-4}	low	stable	++(+)[a]	++(+)[a]	
$MTAP_{5-6}$	pAP_{5-6}	high	stable	+++		
$PHAP_{7-8}$	pAP_{7-8}	high	stable	+++	++	low (+)
$PHAP_{9-10}$	pAP_{9-10}	high	unstable	+		
$LYAY_{1-2}$	pAY_{1-2}	high	stable	+++		
$LYAY_{3-4}$	pAY_{3-4}	high	stable	+++	++++	++
YB_1	pYB_1	high	stable	+++		
YB_2	pYB_2	high	stable	+++	++++	+++
IL2–PE40	pHL906 pHL310	high	stable	+++	+++	low (+)

[a] The activity of $EXAP_{3-4}$ is similar to that of IL2–PE40, taking into consideration its low expression. [b] Stability of expressed proteins was estimated according to Western blot analysis.

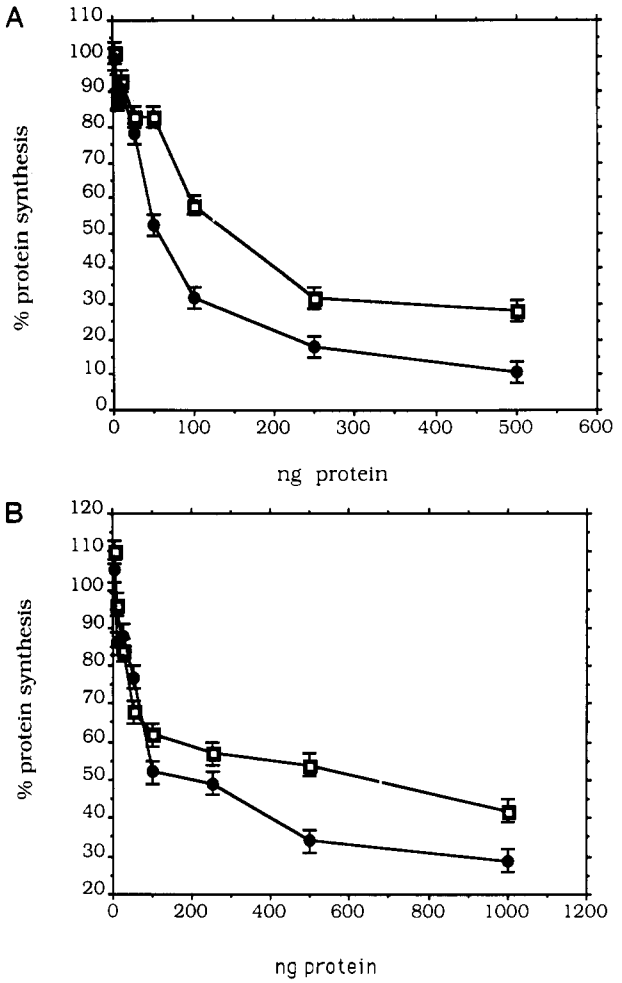

FIGURE 6: Cytotoxic activity of IL2–PE66^{4Glu}(LAP+DUP) on HUT102 cells (A), and on human activated lymphocytes (B). Experiments were performed as described for Figures 4 and 5.

Insertion of the LAP+DUP Sequence into IL2–PE66^{4Glu}. Next, we tested whether insertion of the LAP+DUP sequence would increase the cytotoxicity of another IL2–PE derivative. The LAP+DUP sequence was inserted into IL2–PE66^{4Glu} (Lorberboum-Galski et al., 1990). IL2–PE66^{4Glu} was found to be a much more active cytotoxic molecule for primate and human activated T-cells than IL2–PE40 (Lorberboum-Galski et al., 1990). The new protein, IL2–PE66^{4Glu} (LAP+DUP), was 2–3-fold more cytotoxic to both HUT102 cells (Figure 6A, Table 4) and to activated human lymphocytes (Figure 6B, Table 4). Cytotoxicity on both HUT102 and activated lymphocytes was tested when the different IL2–PE chimeric proteins were added at the same total protein concentration. As slightly different enzymatic activities were measured for the various preparations, the results can be corrected accordingly (Table 3). Thus, IL2–PE66^{4Glu}(LAP+DUP) is even more active (3–5-fold) than IL2–PE66^{4Glu}.

When DNA synthesis on PHA-activated lymphocytes was compared in the presence of IL2–PE(LAP+DUP) derivates, IL2–PE66^{4Glu}(LAP+DUP) was found to be more active than IL2–PE40(LAP+DUP), as originally observed for the authentic IL2–PE molecules that omit the LAP+DUP insertion.

DISCUSSION

Specific and selective agents for the therapy of disorders of the immune response are now being developed on the basis of our increased understanding of the immune response and advances in genetic engineering. Using the chimeric cytotoxin, IL2–PE40, to destroy activated T-cells expressing the IL2 receptor, we previously showed that therapy which targeted IL2 receptor positive cells provided effective and selective immunosuppression. The therapeutic potency of IL2–PE40 had been demonstrated in a number of rodent models (4–10). However, ongoing studies indicate that the rate-limiting step in the cytotoxicity of PE, and most probably of IL2–PE40, is translocation of the protein across membranes to reach the cytosol. The improvement of translocation of chimeric proteins across membranes is, therefore, one of the major goals in developing molecules for the targeted killing of specific cells. Thus, several new chimeric proteins, in which "targeting/sorting" signals were introduced into the IL2–PE40 chimeric gene, were constructed.

Newly designed chimeric proteins were expressed in *E. coli*. Proteins were characterized by gel electrophoresis and Western blot analysis. These proteins were also tested for enzymatic activity in vitro and for cytotoxic activity on a T-cell line that expresses a high level of IL2-R and on human activated T-cells. One of the chimeric proteins, IL2–PE40(LAP+DUP), containing a targeting signal for lysosomal membranes, was 2–3-fold more active than IL2–PE40 when tested on both HUT102 cells and human activated lymphocytes (Figures 4 and 5, Table 4).

One of the approaches reported earlier for increasing the translocation efficiency of an immunotoxin was to expose target cells to an immunotoxin, PE–αTFR (anti-transferrin receptor antibody coupled to PE), and to an adenovirus at the same time. The toxicity of these conjugates, due to the entry through the transferrin receptor, was enhanced 100–300-fold in the presence of adenovirus (Fitzgerald et al., 1983). It was suggested that the enhanced cytotoxicity resulted when adenovirus and the immunotoxins were internalized into the same receptosomes. In the process of infection, adenovirus

enters cells and brings about a virus-mediated disruption of the receptosomes, thus allowing many more immunotoxins to be released into the cytosol than was possible in the absence of the virus.

Although an efficient method, this approach cannot be applied to molecules that might be used for targeted immunotherapy in humans. Therefore, we tried to increase the translocation efficiency of IL2–PE by means of designing new constructs with defined, inserted sequences for targeting/sorting, thus trying to improve the specific cytotoxicity of these molecules. In this situation, improved cytotoxicity will be an integral property of the molecule.

Translocation is mainly the crossing of molecules through biological membranes. Research about the mechanisms involved in translocation has shown that each protein is carrying in its sequence the information for its final target/destination. The targeting signals known today are signals for secretion (Von Heijne, 1985; Boyd & Beckwith, 1990), signals targeting mitochondria (Kemp & Pearson, 1990), targeting signals for lysosomal membranes (Peters et al., 1990), a signal for enodocytosis (Pearse, 1988; Davis et al., 1987; Lazarovits & Roth, 1988; Lobel et al., 1989), and signals targeting endoplasmic reticulum (Bonifacino et al., 1991). The common feature of these sequences is that the chemical characteristics of the signal and not its specific sequence is responsible for the targeting. Fusion of a targeting sequence from one protein to another will change the target of the latter protein.

The existence of a specific molecular mechanism that might be involved in the translocation of PE was suggested before (Jinno et al., 1989). Competition assays have shown that a chimeric protein composed of transforming growth factor α fused to PE, TGFα–PE$^{(Glu57-Asp553)}$, which had reduced binding activity and reduced enzymatic activity but normal translocation efficiency, succeeded in protecting cells from the cytotoxicity of native PE and PE^{Glu57}. In this situation, the reduced binding activity was caused by a mutation at amino acid 57; the reduced enzymatic activity resulted from a mutation at amino acid 553, and the PE^{Glu57} is a mutated PE with a defect in its ability to bind target cells. Because internalization of the molecules was different (TGFα–PE through the receptor to TGF, native PE through its receptor, and PE^{Glu57} through an unspecific mechanism), competition between molecules was most probably at a level different from the entrance of the molecules into the cells.

There is some evidence that the translocation of PE through the receptosome membrane includes two different stages: one necessary for the proteolytic processing of PE and the other for translocation of the processed toxin to the cytosol. The processing of PE occurring at domain II of PE (Ogata et al., 1990) is essential but not sufficient for the cytotoxicity of the molecules. Treatment with different primary amines, such as ammonium chloride which increases pH, protected cells from the cytotoxicity of PE, but had no effect on the correct processing of the molecule. A decrease in pH is most probably needed for a later event—the translocation step.

We therefore inserted a variety of sequences into IL2–PE40. These sequences are known to be involved in targeting/sorting different proteins into specific compartments inside or outside the cell (Table 2). These sequences might be recognized by translocation mechanisms of the receptosome membrane responsible for translocating native PE or by any other mechanism involved. Most of the new chimeric proteins constructed, using a variety of sequences that differ in their chemical and biological characteristics, had either similar or lower cytotoxic activities compared to the authentic IL2–PE40 molecules. Insertion of the new sequences even resulted in a few proteins (EXAP$_{1,2}$, MTAP$_{5,6}$, and PHAP$_{9,10}$) that, although exhibiting full enzymatic activity in vitro, had lost their cytotoxicity toward target cells (Table 5). The loss of cytotoxicity can be ascribed to either the loss of binding affinity to the receptor and/or failure to complete processes after the molecules had been internalized within cells. Neither of these possibilities has been studied in detail here. In contrast, the LAP(+DUP) sequence improved the cytotoxicity of IL2–PE molecules by 2–3-fold.

The LAP sequence is normally found at the cytoplasmic tail of the lysosomal protein, Lysosome Acid Phosphatase (Peters et al., 1990). This protein is processed to a soluble protein after transport to the lysosome. Deletion of the cytoplasmic tail (LAP sequences) or mutations within this sequence prevent internalization of the protein and its accumulation at the cell surface. Moreover, when the LAP sequence was fused to the ectoplasmic and transmembrane domains of hemagglutinin, which is not known to be internalized, the new chimeric protein was internalized very rapidly (Peters et al., 1990). Another chimeric polypeptide, in which the membrane-spanning domain and cytoplasmic tail of LAP were fused to the ectoplasmic domain of the M_r 46 000 mannose 6-phosphate receptor, was rapidly transported to lysosomes, whereas wild-type receptor was not transported to lysosomes (Chaudhary et al., 1990). Thus, the LAP sequence is necessary and sufficient for targeting lysosomes.

The LAP sequence, when placed at the COOH terminus of IL2–PE40, blocked the cytotoxic activity of the chimera [IL2–PE40(LAP)] (Figure 4). These results are compatible with previous observations by Chaudhary et al. (1990), showing complete loss of PE biological activity when its COOH terminus was blocked. Thus, the LAP sequence was unable to restore the activity of the blocked PE.

As in the IL2–PE(LAP+DUP) molecule, where we duplicated the PE COOH terminus (DUP sequence), the increased cytotoxicity of these molecules might be due to the duplication itself, and not due to lysosomal targeting. The chimeric protein, IL2–PE40(DUP), in which we duplicated only the original carboxy terminus of PE, REDLK, omitting the LAP sequence, ruled out this possibility. IL2–PE40(DUP) showed cytotoxic activity similar to that of the authentic IL2–PE40 (Figure 4), thus suggesting that duplication of the COOH terminus of PE in the IL2–PE40 chimera had no effect on its cytotoxic activity toward target cells.

The LAP sequence was shown to enhance the internalization of various proteins containing it (Peters et al., 1990). Therefore, in order to establish that the increased cytotoxicity of IL2–PE40(LAP+DUP) is due to lysosomal targeting and not to the increased efficiency of its internalization, we followed the internalization of IL2–PE40(LAP+DUP), as compared to IL2–PE40, into HUT102 cells. IL2–PE40(LAP+DUP) could be detected within the lysates of target cells, following incubation, at amounts similar to the authentic IL2–PE40 and other IL2–PE40 derivatives. Thus, insertion of the LAP sequence into IL2–PE40 did not enhance its internalization into target cells.

Therefore, our results suggest that the increased cytotoxic activity of IL2–PE40(LAP+DUP) is most probably due to lysosomal targeting. However, we cannot rule out the possibility that the increased cytotoxicity of IL2–PE40-(LAP+DUP) is also due to some other property of the LAP sequence, not identified yet.

Toxins, immunotoxins, and chimeric cytotoxins are internalized into cells via receptor-mediated endocytosis. While

within the cell, most of the molecules are transferred to the lysosomes, degraded or returned to the cell surface, and released into the medium. On the way to the lysosome, very few molecules "escape" and reach the cytosol. The mechanisms by which they do this are not yet understood. Our results suggest that directly targeting the IL2–PE40(LAP+DUP) to the lysosomes allows a higher percentage of those chimeric cytotoxins that are on the way to the lysosome to reach the cytoplasm, thus increasing IL2–PE40(LAP+DUP)'s specific cytotoxicity. As the LAP sequence increased the cytotoxicity of two derivatives of IL2–PE (IL2–PE40 and IL2–PE66^{4Glu}), this sequence might serve as a common sequence for increasing the cytotoxicity of various chimeric proteins. IL2–PE40 was already found to be an effective immunosuppressive agent in models of immune disorders (Bailon et al., 1988; Case et al., 1991; Lorberboum-Galski et al., 1989; Roberge et al., 1989; Kozak et al., 1990; Beraud et al., 1991; Rose et al., 1991). Thus, the improved molecules, IL2–PE40(LAP+DUP) and IL2–PE66^{4Glu}(LAP+DUP), might be used and evaluated as more efficient agents for targeted immunotherapy.

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