Importance of the glutamate residue of KDEL in increasing the cytotoxicity of *Pseudomonas* exotoxin derivatives and for increased binding to the KDEL receptor

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It was previously shown that amino acids 609–613 (REDLK) at the C-terminus of *Pseudomonas* exotoxin (PE) are necessary for cytotoxicity, presumably by directing the toxin to the endoplasmic reticulum (ER) [Chaudhary, Jinno, FitzGerald and Pastan (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 308–312]. Using the anti-[interleukin 2 receptor (IL2R)] immunotoxin anti-Tac(Fv)–PE38 (AT–PE38REDLK), it was found that removing the terminal lysine did not alter the activity, but replacing REDL with KDEL, the most common ER retention sequence, increased activity. To determine which amino acid in KDEL was responsible for the increase in activity, we tested eight C-terminal mutants of AT–PE38REDLK. Using IL2R-bearing MT-1 cells, we found that the glutamate residue of KDEL was required for high activity, as the cytotoxicity of AT–PE38 ending in KDEL, RDEL, KEEL or REEL was much greater than that of AT–PE38 ending in REDL, KEDL, RDDL or KDDL. Using freshly isolated lymphocytic leukaemia cells, AT–PE38 ending in KDEL, REEL or RDEL was more than 100-fold more cytotoxic than AT–PE38 ending in KEDL, REDL, RDDL or the native sequence REDLK. The RDEL sequence also improved the cytotoxic activity of an interleukin 4–PE38 toxin fusion protein. Improved cytotoxic activity correlated with improved binding of the C-termini to the KDEL receptor on rat Golgi membranes. These data indicate that the glutamate residue of KDEL improves the cytotoxicity of PE by increasing binding to a sorting receptor which transports the toxin from the transreticular Golgi apparatus to the ER, where it is translocated to the cytosol and inhibits protein synthesis.

INTRODUCTION

Pseudomonas exotoxin (PE) is a 66 kDa protein toxin that kills cells by inhibition of protein synthesis. Intoxication requires cell binding, toxin internalization, translocation to the cytosol and ADP-ribosylation of elongation factor 2 (EF2). PE is composed of three domains [1], and mutagenesis studies have identified the domains responsible for the different steps of intoxication [2]. Domain Ia (amino acids 1–252) binds to the PE receptor that is present on most animal cells [3]. Domain II (amino acids 253–364) undergoes proteolytic cleavage between arginine-279 and glycine-280, and residues 280–364 are necessary for translocation of the C-terminal toxin fragment (amino acids 280–613) into the cytosol [4,5]. The function of domain Ib (amino acids 365–399) is unknown, and amino acids 365–380, which include a disulphide bond, can be removed without decreasing cytotoxic activity [6,7]. Domain III (amino acids 400–613) contains the ADP-ribosylating activity that inactivates EF2 in the cytosol. The C-terminus REDLK (amino acids 609–613) is not important for ADP-ribosylation activity but is crucial for cytotoxicity [8]. A possible intracellular transport function for this sequence was suggested when it was successfully replaced by the endoplasmic reticulum (ER) retention sequence KDEL [8].

The ER contains several soluble proteins, including chaperones such as BiP and protein disulphide isomerase, which help fold and oxidize proteins in preparation for secretion, but are not themselves secreted [9,10]. The retention of such proteins in the ER is mediated by the ER retention sequence KDEL located at their C-termini [10]. Other retention sequences have been identified, such as HIEL, HYEL, HTEL, RDEL, HVEL, HNEL, HTEL and QEDL in rabbits and rats [11–13], KEEL in mice [14], KQDL in trypanosomes [15], SDEL in *Plasmodium* [16] and HDEL, DDEL and ADEL in yeast [17,18]. KNEL, DKEL and SEHDEL are sequences that also function in mammalian cells [19,20]. Sequences that do not function in mammalian cells include KDELGL, DDEL, KDAS, KDQL, FEHDEL, KDEA and KDEV [9,16,19,20]. ER retention sequences function by returning proteins to the ER in a retrograde fashion after they follow post-ER secretory pathways [21,22]. Calcireticulin is a KDEL-containing protein that has been found to travel at least as far as the transreticular Golgi before returning to the ER [23]. This 'reverse secretory' intracellular routing pathway mediated by the retention sequence is thought to be the pathway by which PE gains access to the ER so that it then can translocate to the cytosol of target cells [8].

When REDLK at the C-terminal sequence of native PE was replaced by KDEL, REDL, RDEL or KEDLK, cytotoxic activity was retained [8]. In subsequent studies using chimaeric toxins which bind to different cellular receptors it was found that the cytotoxicity of toxins containing transforming growth factor α or anti-Tac(Fv) fused to the N-terminus of PE40 (domains II, Ib and III of PE) was increased severalfold by the KDEL mutation [7,24]. Anti-Tac(Fv) is the single-chain-Fv form of the anti-Tac monoclonal antibody to the α-subunit of the interleukin 2 receptor (IL2R). Anti-Tac(Fv)–PE40KDEL, in which anti-Tac(Fv) was fused to PE40 containing KDEL, was 2- to 10-fold

Abbreviations used: PE, *Pseudomonas* exotoxin; ER, endoplasmic reticulum; IL2, interleukin 2; IL2R, IL2 receptor; IL4, interleukin 4; IL4R, interleukin 4 receptor; EF2, elongation factor 2; CLL, chronic lymphocytic leukaemia; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

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more active than anti-Tac(Fv)–PE40 toward most IL2R-bearing cells [7,24]. Moreover, the effect of KDEL was particularly striking on cells from patients with chronic lymphocytic leukaemia (CLL), where cytotoxicity was often increased more than 100-fold [25]. The improvement in cytotoxic activity of anti-Tac(Fv)–PE40KDEL was attributed to either improved binding to an intracellular KDEL receptor that binds either KDEL or REDLK or binding to a receptor that binds KDEL but not REDLK and directs proteins more efficiently to the ER [25].

It is intriguing that relatively conservative amino acid changes are needed to convert the REDLK sequence of PE into the KDEL sequence having improved activity. As removing the terminal lysine does not affect the activity of PE [8], the improved activity on changing from REDL to KDEL might be due to only one of the three alterations, R609K, E610D or D611E. In the present study, we created hybrid sequences between REDL and KDEL at the C-terminus of anti-Tac(Fv)–PE38 and interleukin 4 (IL4)–PE38 to determine which amino acid(s) in REDL must be changed to account for the improved activity of KDEL. We used anti-Tac(Fv)–PE38 rather than anti-Tac(Fv)–PE40 for this analysis because it is about to enter clinical trials. Anti-Tac(Fv)–PE38, abbreviated here to AT–PE38REDLK, differs from anti-Tac(Fv)–PE40 in that much of domain Ib (amino acids 365–380) is missing; this deletion does not diminish cytotoxicity [7]. It has previously been shown that the improved cytotoxic activity of AT–PE38KDEL compared with AT–PE38REDLK was not due to improved binding or improved ADP-ribosylation activity [7]. Thus differences in cytotoxicity due to changes in the C-terminus must be due to a different step required for cell killing, such as intracellular routing.

MATERIALS AND METHODS

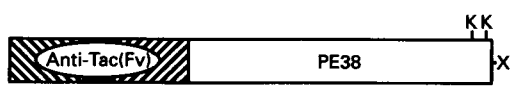
Plasmid construction

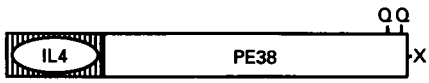
Plasmids pRK79 and pRK749K, encoding AT–PE38REDLK and AT–PE38KDEL respectively, have been described previously [26]. Each plasmid encodes for the C3 connector (ASGGPE) between anti-Tac(Fv) and the toxin. Figure 1 shows these two as well as the new constructions. PCR was performed using 1 min denaturation at 94 °C, 2 min annealing at 55 °C, a 3 min polymerization at 72 °C with a 10 s polymerization extension per cycle and a final 7 min incubation at 72 °C. All plasmids were sequenced using either sequenase 2 (USB, Cleveland, OH, U.S.A.) or a *Taq* Dyedeoxy-Terminator Cycle sequencing kit and an automated sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

For some of the recombinant toxins in this study, an altered form of PE38, termed PE38q, was used. PE38q contains the mutations K590Q and K606Q. A derivative of PE38q ending in REDLR has been useful for chemically conjugating to antibodies, because the lysines normally present in domain III are neither derivatized nor conjugated [27]. These mutations have been shown not to alter cytotoxic activity and in this study PE38q was often used for cloning purposes. We have confirmed that the cytotoxic activities of AT–PE38qKDEL, AT–PE38qRDEL and AT–PE38qREDL are the same as those of AT–PE38KDEL, AT–PE38RDEL and AT–PE38REDL respectively towards MT-1, ATAC-4, HUT-102 and fresh CLL cells (results not shown). Also, the cytotoxicity of AT–PE38qREDLR was equivalent to that of AT–PE38REDLK, confirming that the K613R mutation also does not affect cytotoxicity (results not shown).

pRK79QΔ and pRK79QR, encoding AT–PE38qREDL and AT–PE38qREDLR respectively, were obtained using DNA fragments encoding amino acids 308–613 of PE with mutations of lysine-590 and lysine-606 to glutamine (new base sequence CAA for each codon) and either a deletion of lysine-613 by conversion of AAG into TAG, or a mutation of lysine-613 to arginine by conversion of AAG into CGT [27]. These 1.0 kb *SalI*–*Eco*RI fragments were each ligated to the 3.9 kb *Eco*RI–*SalI* fragment of pRK78 which encodes AT–PE40 [26], resulting in pRK79QΔ and pRK79QR respectively.

pVC4215 and pVC49425 encode PE-REDL and PE-RDEL respectively [8]. To make pRK79RED and pRK79RDE encoding AT–PE38REDL and AT–PE38RDEL respectively the 0.36 kb





X	Protein	Plasmid
KDEL	AT–PE38KDEL	pRK749K
RDEL	AT–PE38RDEL	pRK79RDE
KEEL	AT–PE38KEEL	pRK79KEE
REEL	AT–PE38REEL	pRK79REE
REDL	AT–PE38REDL	pRK79RED
KEDL	AT–PE38KEDL	pRK79KED
KDDL	AT–PE38KDDL	pRK79KDD
RDDL	AT–PE38RDDL	pRK79RDD
REDLK	AT–PE38REDLK	pRK79
REDLR	IL4–PE38qREDLR	pWDMH4.38Q
RDEL	IL4–PE38qRDEL	pRKL4QRD

Figure 1 Schematic of recombinant proteins

Anti-Tac(Fv) and IL4 denote the ligands used for the chimaeric toxins in this study. PE38 denotes the 'C3' connector (ASGGPE) followed by amino acids 253–364 and 381–608 of PE. PE38 contains lysine (native) or glutamate at positions 590 and 606. The amino acids after residue 608 are indicated.

*Bam*HI–*Eco*RI fragment of pVC4215 and pVC49425 were each respectively ligated into the 4.4 kb *Eco*RI–*Bam*HI fragment of pRK79. The 0.36 *Bam*HI–*Eco*RI fragment of these plasmids encodes amino acids 493–608 of PE, followed by the C-terminal residues.

To make pRK79KED, pRK79REE, pRK79RDD, pRK79QRDE and pRK79QK, encoding AT–PE38KEDL, AT–PE38REEL, AT–PE38RDDL, AT–PE38qRDEL and AT–PE38qKDEL respectively, PCR was performed using pRK749K, pRK79, pRK79, prK79QΔ and pRK79QΔ as templates respectively. The primers included VK116 (5′-TGG-CGC-GGT-TTC-TAT-ATC-GCC-3′) and either BK92 (3′-GGG-CCG-TTT-GGC-GGC-TTT-CTT-CTA-GAG-ACT-TAA-GAG-CCG-5′), BK93 (3′-CGG-TTT-GGC-GGC-GCG-CTC-CTC-GAG-ACT-TAA-GAC-GGC-5′), BK94 (3′-GGG-CCG-TTT-GGC-GGC-GCG-CTA-CTG-GAC-ACT-TAA-GAC-GGC-5′), BK58 (3′-GGG-CCG-GTT-GGC-GGC-GCG-CTG-CTC-GAC-ATT-CTT-AAG-GCT-GGC-CGG-CCG-AGG-5′) or BK59 (3′-TCG-GTC-GGG-CCG-GTT-GGC-GGA-TTC-CTG-CTC-GA G-ATT-CTT-AAG-GCT-GGC-CGG-CCG-AGG-5′) respectively. The 0.36 kb *Bam*HI–*Eco*RI fragment of each amplified fragment was then ligated to the 4.4 kb *Eco*RI–*Bam*HI fragment of pRK79, resulting in pRK79KED, pRK79REE, pRKRDD, pRK79QRDE and pRK79QK respectively.

To make pRK79KEE, encoding AT–PE38KEE, pRK79KED was amplified with primers BK181 (5′-CTG-CGG-GTC-TAT-GTG-CCG-CGC-3′) and BK179 (3′-CCG-TTT-GGC-GGC-TTT-CTT-CTC-GAG-ACT-TAA-GCC-5′) and the 0.32 kb *Xho*I–*Eco*RI fragment ligated to the 4.5 kb *Eco*RI–*Xho*I fragment of pRK79KED. To make pRK79KDD, encoding AT–PE38KDDL, pRK749K was amplified with BK181 and BK180 (3′-CCG-TTT-GGC-GGC-TTT-CTG-CTA-GAG-ACT-TAA-GAG-5′) and the 0.32 kb *Xho*I–*Eco*RI fragment ligated to the 4.5 kb *Eco*RI–*Xho*I fragment of pRK749K.

$pWDMH_4 \cdot 38Q$ encodes IL4–PE38qREDLR [28]. This protein contains human IL4 followed by the same toxin portion as AT–PE38qREDLR (Figure 1). pRKL459K or pRKL4QRD, encoding IL4–PE38KDEL or IL4–PE38qRDEL respectively, was obtained by ligating the 0.4 kb *Nde*I–*Hin*dIII fragment of $pWDMH_4 \cdot 38Q$ to the 4.1 kb *Hin*dIII–*Nde*I fragment of either pRK749K or pRK79QRDE respectively. The 0.4 kb *Nde*I–*Hin*dIII fragment encodes IL4, and is identical with the *Nde*I–*Hin*dIII fragment of pWDMH4 [29]. pRKL459, encoding IL4–PE38REDLK, was obtained by ligating the 0.44 kb *Xba*I–*Hin*dIII fragment of $pWDMH_4 \cdot 38Q$ to the 4.1 kb *Hin*dIII–*Xba*I fragment of pRK79. IL4–PE38REDLK and IL4–PE38KDEL are not shown in Figure 1, because their toxin portions are identical with those of AT–PE38REDLK and AT–PE38KDEL respectively.

Plasmid expression and protein purification

Plasmids were transformed into *E. coli* BL21/λDE3 the day before fermentation and grown on Luria broth–ampicillin plates overnight. In the case of pRK749K, pRK79, pRK79QΔ, pRK79QK and pRK79QRDE, the *E. coli* was grown in a 10-litre fermentor with superbroth supplemented with ampicillin (100 mg/l), glucose (0.5%) and $MgSO_4$ (1.62 mM) to an A_{650} of 6–8. Protein expression was induced by the addition of isopropyl thiogalactopyranoside (USB) to 1 mM for 1.5 h. Purified inclusion bodies were obtained from the harvested cell paste by sarcosyl washing, and renatured recombinant protein was purified with the aid of an IL2R-affinity chromatography column as reported previously [30]. In the case of the other plasmids, recombinant protein was purified by a protocol similar to that reported previously [31]. *E. coli* was grown in 1 litre of supplemented superbroth to an A_{650} of 2.0, and induced with isopropyl thiogalactopyranoside (1 mM) for 1.5 h.

The harvested cells were resuspended in 160 ml of TES (50 mM Tris/HCl, pH 8.0, 20 mM EDTA and 0.1 M NaCl) and treated with lysozyme (33 mg) for 30 min followed by DNAase (0.4 mg) for 30 min. Pure inclusion bodies were obtained from the centrifuged pellet by three to four cycles of resuspension in 180 ml of TES + 2.5% Triton X-100 followed by centrifugation. The pelleted inclusion bodies were worked by four cycles of resuspension in 180 ml of TES and centrifugation. All resuspensions were performed at 22 °C using a tissuemizer (Thomas Scientific, Swedesboro, NJ, U.S.A.). The inclusion body pellet was mixed to a slurry with 1–2 ml of TES and dissolved by sonication after the addition of 7 ml of denaturation buffer (7 M guanidine containing 0.1 M Tris/HCl, pH 8.0, and 2 mM EDTA). The approx. 100 mg of denatured protein was adjusted to 10 mg/ml by the addition of denaturation buffer and reduced by the addition of dithioerythritol (Sigma, St. Louis, MO, U.S.A.) to a final concentration of 65 mM for 10–24 h at 22 °C. The reduced protein was refolded by rapid (20 s) 100-fold dilution into a cold stirring redox buffer containing 0.1 M Tris/HCl, pH 8.0, 2 mM EDTA, 0.5 M L-arginine and 0.9 mM GSSG (Sigma). The clear solution was incubated at 10 °C for 36 h and dialysed against 0.1 M urea in 0.02 M Tris/HCl, pH 7.4, to an ionic strength less than that of 60 mM NaCl. The filtered renatured protein was then purified by Q-Sepharose, Mono Q and size-exclusion chromatography as reported previously [32]. The monomeric protein was more than 95% homogeneous as ascertained by SDS/PAGE (results not shown). Several recombinant immunotoxins, including AT–PE38REDLK and AT–PE38KDEL, were purified by both methods and the purification method did not affect purity or cytotoxic activity (results not shown). Protein was quantified by the Pierce Coomassie Plus Bradford assay (Rockford, IL, U.S.A.) using BSA as a standard, a method that has been shown to produce the same results as amino acid analysis of PE-derived immunotoxins [24].

Cytotoxicity assay

ATAC-4 cells are A431 human epidermoid carcinoma cells that have been transfected with a plasmid encoding the human IL2R α-subunit [26]. They were plated in 150 μl aliquots of 1.5×10^4 cells in Dulbecco's modified Eagles medium (DMEM) containing 5% fetal bovine serum (FBS) in 96-well plates 24 h before toxin addition. Suspensions of cells (HUT-102, MT-1 and Daudi) were plated in 50 μl aliquots of 4×10^4 cells in RPMI containing 10% FBS in 96-well plates just before toxin addition. Different concentrations of recombinant toxins diluted in 50 μl of the same cell culture medium were added to the cells. After incubation for 20–24 h, the cells were labelled with [^{3}H]leucine (1 μCi/well) for 3–6 h. Incorporated protein was then harvested on to glass-fibre filters and quantified in a Betaplate scintillation counter (Pharmacia–LKB, Piscataway, NJ, U.S.A.). The IC_{50} values were the calculated concentrations necessary for 50% inhibition of protein synthesis. Cells from a patient with the prolymphocytic variant of CLL (patient one in ref. [25]) were isolated and incubated with toxins as described previously [25], except that interleukin 7 (10 ng/ml) was added to the leucine-poor media to improve survival during the 60 h incubation with toxins. All experiments were performed in triplicate. The median of each triplicate, which usually differed from the other two results by less than 10%, was used for determination of IC_{50}.

KDEL-binding assay

Golgi membranes were purified and the KDEL-binding assay was performed essentially as previously described [20,33] with some modifications. Specifically, 128 g of liver from 11 rats was minced and homogenized using a tissuemizer with 500 ml of 0.5 M sucrose. All sucrose solutions contained 0.05 M sodium maleate, pH 6.5. The homogenate was centrifuged at 600 *g* (2000 rev./min) in a GSA rotor (Sorvall, Wilmington, DE, U.S.A.) for 10 min, and the thick opaque supernatant collected by aspiration. Using about one-half of the supernatant, 27 ml was layered over 10 ml of 1.3 M sucrose in each of twelve 25 mm × 89 mm polyallomer centrifuge tubes (Beckman, Palo Alto, CA, U.S.A.), and centrifuged at 63000 *g* (21700 rev./min) for 2 h in an SW28 rotor (Beckman). The tan layer over the sucrose was aspirated in a total volume of 40 ml and adjusted to 1.1 M sucrose by the addition of 120 ml of 1.3 M sucrose. Using about 70% of this mixture, each of six 25 mm × 89 mm polyallomer tubes were prepared containing layers of 7.6 ml of 1.25 M sucrose, 19 ml of Golgi fraction, 6.7 ml of 1 M sucrose and 4.8 ml of 0.5 M sucrose. After centrifugation at 87200 *g* (25000 rev./min) for 90 min in a SW28 rotor, the tan–white flakes were removed from between the top two layers. The 16 ml of aspirate was diluted with 16 ml of 0.8 M NaCl and centrifuged at 165000 *g* (50000 rev./min) for 30 min in a Ti50 rotor (Beckman). The pellets were resuspended in approx. 1.5 ml of 0.1 M Na_2CO_3 for 10 min at 4 °C, centrifuged in a Microfuge at 11000 *g* (12000 rev./min) for 10 min, resuspended in approx. 1.5 ml of 0.1 M Hepes/KOH, pH 7.5, for 10 min, and after recentrifugation resuspended in 0.01 M Hepes/KOH, pH 7.5. Total Golgi membrane protein, measured like the immunotoxins, was 1.72 mg or approx. 440 μg per rat.

To measure binding to the KDEL receptor, the peptides YTSE-KDEL, YTSE-REDLK, YTSE-REDL, YTSE-RDDL, YTSE-KEDL, YTSE-RDEL, YTSE-KEEL, YTSE-REEL and YTSE-KDDL were synthesized by Genosys (The Woodlands, TX, U.S.A.) and CPGKPPKDEL was synthesized by Dr. Lively at Bowman Gray School of Medicine (Winston-Salem, NC, U.S.A.). YTSE-KDEL was iodinated using lactoperoxidase (Enzymobeads; Bio-Rad, Richmond, CA, U.S.A.) and purified on a PD-10 column (Pharmacia, Piscataway, NJ, U.S.A.). Binding reaction mixtures each contained 1 μg of Golgi membrane protein, 1 ng (30000 c.p.m.) of ^{125}I-YTSE-KDEL, 0.05 M sodium cacodylate (Sigma), 0.02 M NaCl, 250 μg/ml BSA, 0.01% (v/v) Triton X-100 (Sigma) and different concentrations of competing molecules. After incubation of the 25 μl reaction mixtures in 1.8 ml Microfuge tubes on ice for 10–20 min, the tubes were centrifuged at 11000 *g* for 5–8 min in the cold. The location of invisible Golgi pellet in the Microfuge tube could be predicted by its placement in the rotor, allowing nearly complete removal of the supernatant by aspiration. Golgi pellets were then counted in a Beckman γ-counter.

RESULTS

The activity of PE increases when its C-terminus, REDLK, is replaced with KDEL, but is unchanged when it is replaced with REDL [7,8,24]. To determine which amino acid change causes the improved cytotoxic activity, chimaeric toxins targeting IL2R- or IL4R-bearing cells were designed with hybrid C-termini. Chimaeric toxins containing anti-Tac(Fv) or IL4 as ligands and the C-terminus REDLK, REDL, KDEL, KEDL, RDDL, REEL, RDEL, KEEL, KDDL or REDLR were studied using cytotoxicity assays on target cells and KDEL-receptor-binding assays. Schematic diagrams of the toxins are shown in Figure 1.

Effect of the C-terminus on the cytotoxicity of AT–PE38 on MT-1 cells

To determine which amino acid(s) of the PE C-terminal REDL sequence must be changed to obtain the high activity of the KDEL sequence, AT–PE38KDEL mutants containing hybrid sequences were tested against the ATL cell line MT-1. This cell line was previously found to display a 4–10-fold difference in sensitivity to AT–PE38REDLK and AT–PE38KDEL [7]. As shown in Table 1, the IC_{50} of AT–PE38REDLK varies from 0.1 to 0.6 ng/ml in different experiments, but in each assay the IC_{50} is severalfold higher than that of AT–PE38KDEL. With removal of lysine-613 from AT–PE38REDLK, the resulting molecule, AT–PE38REDL, has the same cytotoxic potency. Thus one or more of the three mutations R609K, E610D and D611E is responsible for the increased cytotoxicity of AT–PE38KDEL. Each of these mutations was examined separately by testing AT–PE38KEDL, AT–PE38RDDL and AT–PE38REEL. As shown in Table 1 and Figure 2(a), AT–PE38REEL was as cytotoxic as AT–PE38KDEL. AT–PE38RDEL and AT–PE38KEEL also had the same cytotoxic activity as AT–PE38KDEL (Table 1), indicating that D611E was the mutation that resulted in the improved activity of the KDEL sequence. The R609K and E610D mutations alone had no effect because AT–PE38KEDL, AT–PE38RDDL and AT–PE38KDDL each displayed less than 25% of the cytotoxic activity of AT–PE38KDEL (Table 1).

Sensitivity of CLL cells to changes in the C-terminus

To examine the effect of the retention-sequence mutations on cells that respond to AT–PE38KDEL but not to AT–PE38REDLK, we tested the AT-containing toxins on fresh cells from a patient with CLL. Cells from many of such patients are very sensitive to AT–PE40KDEL but very insensitive to AT–PE40 [25]. Table 2 shows that the CLL cells were very sensitive to immunotoxins ending in KDEL, REEL and RDEL, but were not sensitive to those ending in REDLK, REDL, KEDL and RDDL. The cytotoxicity curves of some of these recombinant toxins are depicted in Figure 2(b). The data support the hypothesis that the increased cytotoxic activity by KDEL compared with REDL is due to D611E but not E610D or R609K. Furthermore, the effect of the high-activity sequence on such cells is more than 100-fold, compared with only 4–10-fold on MT-1 cells.

Sensitivity of HUT-102 and ATAC-4 cells to changes in the retention sequence

We examined two other cell types to determine whether the D611E mutation was also important in such cells. Table 3 shows that the transfected cell line ATAC-4 and the ATL line HUT-102, both of which display IL2Rs, are also more sensitive to toxins ending in KDEL, REEL, KEEL and RDEL than to those ending in REDLK, REDL, KEDL, KDDL or RDDL. In this case the increase in sensitivity was usually less than 3-fold. Nevertheless, these results further demonstrate the importance of the D611E mutation in improving the cytotoxic activity of PE.

Effect of the RDEL retention sequence in IL4 toxin

To determine whether the increase in cytotoxicity caused by the RDEL C-terminus was confined to toxins containing anti-Tac(Fv), we made IL4–PE38qRDEL and compared it in cytotoxicity assays with IL4–PE38qREDLR [28], as well as with

Table 1 Effect of C-terminus of AT–PE38 on cytotoxicity toward MT-1 cells

MT-1 cells (4×10^4) were incubated with toxins in 100 μl aliquots of DMEM + 5% FBS for 20–24 h at 37 °C. The cells were pulsed with [^{3}H]leucine (1 μCi/well) for 3–5 h, harvested, and the incorporated protein was quantified. Results for percentage cytotoxicity relative to KDEL are means ± S.D.

Immunotoxin	C-Terminus	IC_{50} (ng/ml) Assay 1	Assay 2	Assay 3	Cytotoxicity relative to KDEL (%)
AT–PE38KDEL	KDEL	0.095	0.095	0.025	100
AT–PE38RDEL	RDEL	0.09	0.08	0.025	108 ± 12
AT–PE38KEEL	KEEL	0.095	0.095	0.03	96 ± 6
AT–PE38REEL	REEL	0.09	0.15	0.025	87 ± 22
AT–PE38REDL	REDL	0.3	0.55	0.1	25 ± 7
AT–PE38KEDL	KEDL	0.35	0.45	0.11	24 ± 3
AT–PE38KDDL	KDDL	0.7	0.55	0.12	17 ± 4
AT–PE38RDDL	RDDL	0.35	0.6	0.11	22 ± 5
AT–PE38REDLK	REDLK	0.35	0.6	0.11	22 ± 6

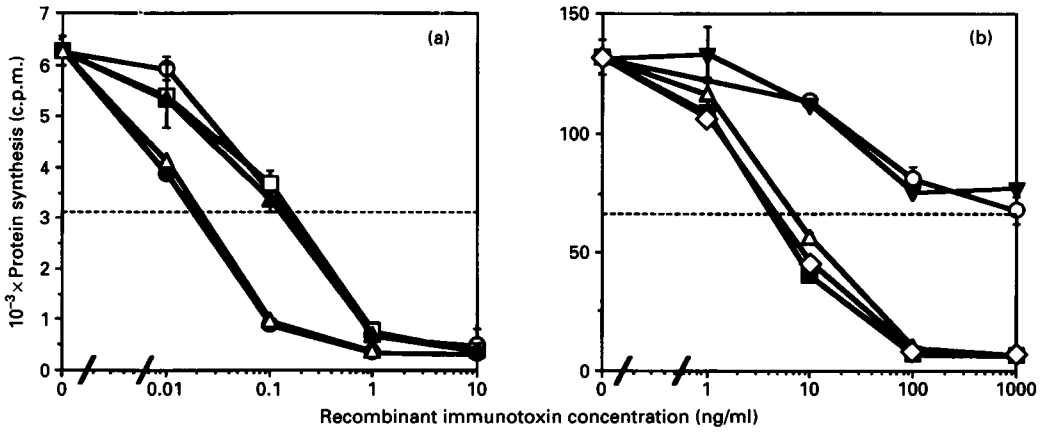


Figure 2 Cytotoxic activity of AT–PE38 mutants differing in retention sequence

Cytotoxicity toward MT-1 cells is shown in (**a**) for AT–PE38REDL (○), AT–PE38KEDL (□), AT–PE38RDDL (▲), AT–PE38REEL (●) and AT–PE38KDEL (△). Cytotoxicity toward fresh cells from a patient with CLL is shown in (**b**) for AT–PE38qREDL (○), AT–PE38KDEL (△), AT–PE38REDLK (▼), AT–PE38qKDEL (◇) and AT–PE38qRDEL (■). The dotted line indicates 50% inhibition of protein synthesis. Error bars show S.D. values, and are not visible if less than the size of the point marker.

Table 2 Effect of retention sequence on cytotoxicity toward fresh CLL cells

Aliquots (0.1 ml; 1×10^5 cells) in media containing 88% leucine-free RPMI, 2% RPM1, 10% FBS and 10 ng/ml interleukin 7 were incubated with different concentrations of immunotoxins for 60 h, labelled with [^{3}H]leucine for 6–8 h, and harvested. The IC_{70} was the calculated toxin concentration necessary for 70% inhibition of protein synthesis. IC_{70} values were used to compare the immunotoxins because in some cases the concentrations of protein used were inappropriate for determination of IC_{50} values. This assay was independent of the one shown in Figure 2(b).

Immunotoxin	C-Terminus	IC_{70} (ng/ml)
AT–PE38REDLK	REDLK	> 1000
AT–PE38REDL	REDL	> 1000
AT–PE38KDEL	KDEL	3.5
AT–PE38KEDL	KEDL	> 1000
AT–PE38RDDL	RDDL	> 1000
AT–PE38REEL	REEL	6
AT–PE38RDEL	RDEL	5

Table 3 Cell lines that were less sensitive to changes in retention sequence

Cytotoxicity assays were performed as in Tables 1 and 2. IC_{50} values are expressed as means ± S.D. of several independent assays.

Immunotoxin	C-Terminus	IC_{50} (ng/ml) ATAC-4	HUT-102
AT–PE38KDEL	KDEL	0.02 ± 0.004	0.04 ± 0.008
AT–PE38RDEL	RDEL	0.02 ± 0.007	0.04 ± 0.004
AT–PE38KEEL	KEEL	0.019 ± 0.003	0.035 ± 0.004
AT–PE38REEL	REEL	0.014 ± 0.001	0.045 ± 0.02
AT–PE38REDL	REDL	0.07 ± 0.05	0.07 ± 0.03
AT–PE38KEDL	KEDL	0.045 ± 0.02	0.06 ± 0.02
AT–PE38KDDL	KDDL	0.05 ± 0.001	0.075 ± 0.02
AT–PE38RDDL	RDDL	0.06 ± 0.03	0.065 ± 0.02
AT–PE38REDLK	REDLK	0.08 ± 0.03	0.08 ± 0.02

Table 4 Recombinant IL4 toxins with increased activity

ATAC-4 cells were plated in 96-well plates (1.5×10^4/well) 24 h before addition of toxins, and then incubated with toxins for 20–24 h in 200 μl of media (DMEM + 5% FBS) before labelling with [^{3}H]leucine as in Table 1. Daudi cells were plated in 96-well plates (4×10^4/well) just before the addition of toxins, and then incubated with toxins in 100 μl of media (RPMI + 10% FBS).

		IC_{50} (ng/ml)			
		ATAC-4		Daudi	
Immunotoxin	C-Terminus	Assay 1	Assay 2	Assay 1	Assay 2
IL4–PE38qREDLR	REDLR	3.0	3.5	13	30
IL4–PE38REDLK	REDLK	3.9	3	8	25
IL4–PE38qRDEL	RDEL	0.45	0.8	3.2	5.5
IL4–PE38KDEL	KDEL	0.4	0.4	2.7	4

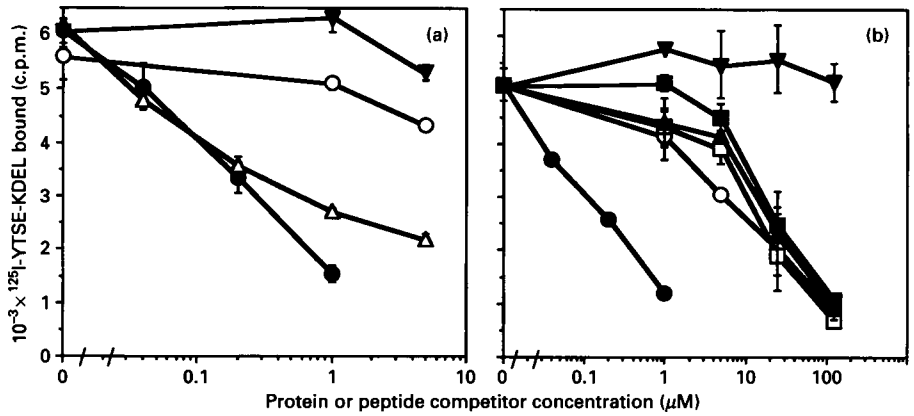


Figure 3 Binding of immunotoxins and peptides to the KDEL receptor

Purified Golgi membranes from rat liver (1 μg) were incubated in 25 μl aliquots with 1 ng of ^{125}I-YTSE-KDEL (about 30000 c.p.m.) combined with different concentrations of unlabelled YTSE-KDEL (●) in (**a**) and (**b**), the immunotoxins AT–PE38KDEL (△), AT–PE38REDL (○) or AT–PE38REDLK (▼) in (**a**) or the peptides YTSE-REDL (○), YTSE-KEDL (□), YTSE-RDDL (▲), YTSE-KDDL (■) or YTSE-REDLK (▼) in (**b**). Error bars are as in Figure 2.

IL4–PE38REDLK and IL4–PE38KDEL. The REDLR C-terminus is equivalent to that of REDLK and REDL, as the cytotoxicity of AT–PE38qREDLR is equivalent to that of AT–PE38REDLK and AT–PE38qREDL (results not shown). As target cells for IL4 toxins, we used ATAC-4, an A431 derivative which expresses IL2Rs but has the same IL4-toxin sensitivity as A431 cells ([7,26]; results not shown). Another target was the IL4R-bearing Burkitt's lymphoma line Daudi. Table 4 shows that IL4–PE38qRDEL or IL4–PE38KDEL was 4–10-fold more cytotoxic than IL4–PE38qREDLR or IL4–PE38REDLK toward ATAC-4 cells, and 2.5–7.5-fold more cytotoxic toward Daudi cells. Thus the improved cytotoxicity imparted by the mutation D611E is independent of the ligand directing the toxin to target cells.

Binding of the C-terminus to the KDEL receptor

Although circumstantial evidence indicated that PE or its mutants bind to the KDEL receptor, direct experimental evidence of this was lacking up until now. If the improved cytotoxicity imparted by the D611E mutation is due to improved transfer of the active toxin fragment from the endocytic vesicle through the transreticular Golgi to the ER, where it could translocate to the cytosol, it would be expected that toxins containing glutamate-611 would bind better to the KDEL receptor than those toxins without this mutation. To determine if this is the case, we tested the recombinant immunotoxins for binding to the KDEL receptor. To do this, we utilized an assay in which peptides are used to compete for the binding of radiolabelled KDEL-containing peptide to the KDEL receptor on purified Golgi membranes from rat liver [20]. As shown in Figure 3(a), AT–PE38KDEL was effective at competing for the binding of ^{125}I-YTSE-KDEL to Golgi membranes, whereas AT–PE38REDL and AT–PE38REDLK showed little or no displacement of ^{125}I-YTSE-KDEL.

Below 0.1 μM, the competition by AT–PE38KDEL was equivalent to that of unlabelled YTSE-KDEL (Figure 3). Furthermore, a peptide of sequence CPGKPPKDEL, which contains the last nine amino acids of AT–PE38KDEL, showed ^{125}I-YTSE-KDEL displacement similar to that of YTSE-KDEL (results not shown). At higher concentrations, the competitive effect of AT–PE38KDEL reached a plateau, which could be due to the bulky protein moiety interfering with binding. Accordingly, to evaluate the binding of the other recombinant immunotoxins, we compared their EC_{45} values, the immunotoxin concentrations necessary for 45% competition of ^{125}I-YTSE-KDEL binding. As shown in Table 5, the immunotoxins with high activity (AT–PE38KDEL, AT–PE38RDEL, AT–PE38KEEL and AT–PE38REEL) had EC_{45} values of 2.5 μM or less, whereas the immunotoxins with lower activity (AT–PE38KEDL, AT–

Table 5 Binding of immunotoxins to the rat liver KDEL receptor

EC_{45} is the concentration of protein necessary to displace ^{125}I-YTSE-KDEL by 45% from rat Golgi membranes during a 10–20 min incubation at 4 °C. The EC_{45} values shown are the means ± S.D. of duplicate independent experiments. Concentrations were kept to a maximum of 5 μM to prevent aggregation and precipitation under the conditions of the assay. The relative affinity of AT–PE38KDEL is set to 100%.

Immunotoxin	EC_{45} (μM)	Relative affinity (%)
AT–PE38KDEL	0.42 ± 0.2	100
AT–PE38RDEL	0.31 ± 0.15	135
AT–PE38KEEL	1.85 ± 0.3	23
AT–PE38REEL	2.5 ± 0.2	17
AT–PE38REDL	> 5	< 8
AT–PE38KEDL	> 5	< 8
AT–PE38KDDL	> 5	< 8
AT–PE38RDDL	> 5	< 8
AT–PE38REDLK	> 5	< 8

Table 6 Binding of peptides to the KDEL receptor

The peptides were tested for KDEL receptor binding as in Table 5. The affinity of YTSE-KDEL was set to 100%.

Peptide sequence	EC_{45} (μM)	Relative affinity (%)
YTSE-KDEL	0.18 ± 0.04	100
YTSE-RDEL	0.17 ± 0.09	105
YTSE-KEEL	0.5 ± 0.05	35
YTSE-REEL	1.5 ± 0.2	12
YTSE-REDL	13 ± 7.5	1.4
YTSE-KEDL	13 ± 1.5	1.4
YTSE-KDDL	27 ± 11	0.7
YTSE-RDDL	17 ± 0.8	1.1
YTSE-REDLK	> 625	< 0.03

PE38KDDL, AT–PE38REDL, AT–PE38RDDL and AT–PE38REDLK) showed insignificant displacement at 5 μM. Immunotoxin aggregation made assessment of higher concentrations unreliable. In the binding assays the KDEL and RDEL sequences were 4–8-fold more potent in displacing ^{125}I-YTSE-KDEL than the KEEL and REEL sequences. Thus, not only was the D611E mutation of PE required for significant KDEL receptor binding, but the E610D mutation also appeared important for optimal binding. The R609K mutation was not important, since the binding of AT–PE38RDEL did not differ from that of AT–PE38KDEL. The modest improvement in binding with the E610D mutation was not seen in the cytotoxicity assays.

To quantify the binding of the lower-activity immunotoxins, we tested peptides containing the altered C-termini. We chose peptides YTSE-KDEL, YTSE-RDEL, YTSE-KEEL, YTSE-REEL, YTSE-REDL, YTSE-KEDL, YTSE-RDDL, YTSE-KDDL and YTSE-REDLK, that could be tested in much higher concentrations than their respective immunotoxins. Figure 3(b) and Table 6 show that all the low-activity octomeric peptides were similarly effective in displacing ^{125}I-YTSE-KDEL from the KDEL receptor, having 0.7–1.4% of the affinity of unlabelled YTSE-KDEL. In contrast, YTSE-REDLK showed no significant competition at 125 μM (Figure 3b) or 625 μM (Table 6). As YTSE-KDEL displayed significant competition at 0.04 μM, it was over 15000-fold more potent than YTSE-REDLK. Table 6 also shows that YTSE-RDEL had the same high activity as YTSE-KDEL, and that YTSE-KEEL and YTSE-REEL bound with 3- to 8-fold less affinity than YTSE-KDEL. These results are similar to those obtained by testing the four high-activity immunotoxins. Thus whereas the cytotoxic assays divided the molecules into two activity classes depending only on the D611E mutation, KDEL-receptor-binding assays divided them into four classes: (1) high affinity with both the E610D and D611E mutations, (2) 12–35% affinity with only the D611E mutation, (3) approx. 1% affinity with neither mutation and (4) undetectable binding with the native C-terminus of PE, which contains a lysine after REDL.

DISCUSSION

Our goal in the present study was to determine which amino acid mutations of the native PE C-terminus REDLK are required for the increased cytotoxic activity observed when REDLK (or its equivalent sequence REDL) is replaced with the ER retention sequence KDEL, and to correlate cytotoxic effects with KDEL receptor binding. We found that D611E but not R609K or E610D was required to increase cytotoxic activity. The improvement in cytotoxic activity resulting from the D611E mutation ranged from severalfold to more than 100-fold depending on the target cell used. The increased cytotoxicity was observed when either IL2 or IL4 receptors were targeted. In binding studies with rat liver Golgi membranes, all molecules ending in leucine were found to bind to the KDEL receptor, with the D611E mutation being of major importance and the E610D mutation being of minor importance.

A hypothesis for the function of the C-terminus of PE

The present series of experiments are consistent with a model whereby PE is proteolytically cleaved after internalization, the terminal lysine residue is removed, and the resulting 37 kDa C-terminal active fragment is transported from the endocytic compartment through the transreticular Golgi to the ER, in a fashion similar to that by which proteins such as calcireticulin, protein disulphide isomerase and BiP are returned from post-secretory pathways to the ER [21–23]. This hypothesis was suggested by several previous experiments showing that the C-terminus of PE does not play a role in other steps that are necessary for cytotoxicity, which include (1) binding of cell surface receptors [7,34], (2) formation by proteolytic cleavage of a 37 kDa fragment containing the enzyme and translocating functions [4,5], (3) ADP-ribosylation activity [8] and (4) transport of the 37 kDa fragment of PE by microsomes (a possible model for the translocation of PE to the cytosol) [35]. Thus the 37 kDa C-terminal fragment of PE probably uses the KDEL receptor to move to the ER, from which compartment it may be translocated to the cytosol to ADP-ribosylate EF2.

Correlation of cytotoxicity with KDEL receptor binding

We found that the immunotoxins AT–PE38KDEL, AT–PE38RDEL, AT–PE38REEL and AT–PE38KEEL, which had the highest cytotoxicity, also exhibited the highest binding affinity to the KDEL receptor. This finding strengthens the hypothesis that these immunotoxins are transported by the KDEL receptor to the ER, from which they are translocated to the cytosol. We found that our immunotoxins at high concentrations were less able than peptides to block completely the binding of ^{125}I-YTSE-KDEL to Golgi membranes (Figure 3). To our knowledge, proteins have not been used previously in assays of this sort. One possible explanation for decreased competition is that the

immunotoxin aggregates at high concentrations and at low pH during the assay. However, the same 30–40% background was observed when PE35KDEL, the active fragment of AT–PE38KDEL, which has a lower isoelectric point and is very stable under such conditions (results not shown), was used. We assume that, as the interaction of KDEL with the KDEL receptor is weak (reported K_d up to 0.2 μM) [20], the acidic residues at the C-termini may also interact intramolecularly or intermolecularly with residues in PE or the targeting ligand. Such interactions are not present when peptides are tested.

Another observation was that, whereas the REDL and REDLK sequences imparted equal cytotoxicity, the terminal lysine of PE completely prevented binding to the KDEL receptor. This suggests that either native PE does not really bind to the KDEL receptor or a cellular carboxypeptidase removes the lysine residue before KDEL receptor binding. We favour the latter explanation, because immunotoxins ending in REDLR are just as active as those ending in REDLK or REDL ([27] and Table 4).

Differences among cells in sensitivity to changes in the ER retention sequence

It remains unknown why different types of cell show different sensitivities to changes in the ER retention sequence of the immunotoxins, and why the cytotoxic activity of each immunotoxin was not strictly proportional to its binding affinity to the KDEL receptor. We speculate that binding affinity to the KDEL receptor is only one of many variables that determine how efficiently the KDEL receptor can transport the protein intracellularly. For example, if the cell surface receptor for an immunotoxin is present in high numbers or is very rapidly internalized, the concentration of toxin exposed to the KDEL receptor may be much higher than the K_d for REDL binding, which could minimize the difference in cytotoxicity between the high- and low-activity toxins. Another possibility is that the transport rate of the KDEL receptor differs in different cells. If fewer molecules of AT–PE38REDL than AT–PE38KDEL bind to the KDEL receptor, but the receptor transports and recycles quickly, the two immunotoxins may exhibit equal cytotoxicity. Thus differences in binding affinity for the KDEL receptor could overestimate differences in cytotoxic activity.

It is possible that the KDEL receptor in the rat Golgi membranes tested is different from that in the tumour cells. It has previously been shown that the yeast HDEL receptor, encoded by the ERD2 gene, recognizes HDEL or DDEL in *Kluyveromyces lactis* but only HDEL in *Saccharomyces cerevisiae* [17,36]. Furthermore, a point mutation within the receptor of *K. lactis* abolished recognition of HDEL but not DDEL [37]. Recently two different human receptors that bind KDEL have been cloned [38,39]. It is possible that differences in expression of these and other KDEL receptors in different cell types account for the differences in sensitivity among various cell types to changes in the C-terminus of PE, including the more than 100-fold difference in sensitivity between high- and low-activity immunotoxins on CLL cells.

Quantifying intracellular transport

The ability of cytotoxic immunotoxins to prevent ^{125}I-YTSE-KDEL from binding to its receptor emphasizes their potential use in quantifying intracellular localization mediated by such receptors. Previously, protein disulphide isomerase molecules ending in KDEL, KEDL, RDEL, REEL, REDL, KEEL, KDDL or RDDL were shown to be secreted from COS cells, with minimal secretion being approx. 15% with KDEL, and maximal secretion being about 45% with REDL [40]. Unlike our results, however, increased retention within the cell was observed after substitution of REDL with either KEDL or RDDL. Also unlike our results, substitution of REDL with REEL was not as effective as substitution of REDL with KDEL. Nevertheless, the glutamate residue of the KDEL sequence in protein disulphide isomerase was very important for the function of the ER retention sequence [40]. Discrepancies in results from studies of protein secretion and protein cytotoxicity may be due to the higher sensitivity of the latter approach in measuring retrograde protein transport, or to other rate-limiting factors.

In summary, we have constructed mutants of the recombinant immunotoxins AT–PE38REDLK and IL4–PE38REDLK which contain hybrid ER retention sequences. Our results show that the mutation D611E is required to convert REDL into the high-activity form.

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REFERENCES

1 Allured, V. S., Collier, R. J., Carroll, S. F. and McKay, D. B. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1320–1324
2 Hwang, J., FitzGerald, D. J., Adhya, S. and Pastan, I. (1987) Cell **48**, 129–136
3 Kounnas, M. Z., Morris, R. E., Thompson, M. R., FitzGerald, D. J., Strickland, D. K. and Saelinger, C. B. (1992) J. Biol. Chem. **267**, 12420–12423
4 Ogata, M., Fryling, C. M., Pastan, I. and Fitzgerald, D. J. (1992) J. Biol. Chem. **267**, 25396–25401
5 Ogata, M., Chaudhary, V. K., Pastan, I. and FitzGerald, D. J. (1990) J. Biol. Chem. **265**, 20678–20685
6 Siegall, C. B., Chaudhary, V. K., FitzGerald, D. J. and Pastan, I. (1989). J. Biol. Chem. **264**, 14256–14261
7 Kreitman, R. J., Batra, J. K., Seetharam, S., Chaudhary, V. K., FitzGerald, D. J. and Pastan, I. (1993) Bioconjug. Chem. **4**, 112–120
8 Chaudhary, V. K., Jinno, Y., FitzGerald, D. and Pastan, I. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 308–312
9 Munro, S. and Pelham, H. R. B. (1987) Cell **48**, 899–907
10 Pelham, H. R. B. (1989) Annu. Rev. Cell Biol. **5**, 1–23
11 Robbi, M., Beaufay, H. and Octave, J.-N. (1990) Biochem. J. **269**, 451–458
12 Medda, S. and Proia, R. L. (1992) Eur. J. Biochem. **206**, 801–806
13 Fliegel, L., Newton, E., Burns, K. and Michalak, M. (1990) J. Biol. Chem. **265**, 15496–15502
14 Mazzarella, R. A., Srinivasan, M., Haugejorden, S. M. and Green, M. (1990) J. Biol. Chem. **265**, 1094–1101
15 Hsu, M. P., Muhich, M. L. and Boothroyd, J. C. (1989) Biochemistry **28**, 6440–6446
16 Pelham, H. R. B. (1990) Trends Biochem. Sci. **15**, 483–486
17 Lewis, M. J., Sweet, D. J. and Pelham, H. R. B. (1990) Cell **61**, 1359–1363
18 Pidoux, A. L. and Armstrong, J. (1992) EMBO J. **11**, 1583–1591
19 Andres, D. A., Dickerson, I. M. and Dixon, J. E. (1990) J. Biol. Chem. **265**, 5952–5955
20 Wilson, D. W., Lewis, M. J. and Pelham, H. R. B. (1993) J. Biol. Chem. **268**, 7465–7468
21 Pelham, H. R. B. (1988) EMBO J. **7**, 913–918
22 Dean, N. and Pelham, H. R. B. (1990) J. Cell Biol. **111**, 369–377
23 Peter, F., Van, P. N. and Soling, H. (1992) J. Biol. Chem. **267**, 10631–10637
24 Seetharam, S., Chaudhary, V. K., FitzGerald, D. and Pastan, I. (1991) J. Biol. Chem. **266**, 17376–17381
25 Kreitman, R. J., Chaudhary, V. K., Kozak, R. W., FitzGerald, D. J. P., Waldmann, T. A. and Pastan, I. (1992) Blood **80**, 2344–2352
26 Kreitman, R. J., Bailon, P., Chaudhary, V. K., FitzGerald, D. J. P. and Pastan, I. (1993) Blood **83**, 426–434
27 Debinski, W. and Pastan, I. (1992) Cancer Res. **52**, 5379–5385
28 Debinski, W., Puri, R. K. and Pastan, I. (1994) Int. J. Cancer **58**, 744–748
29 Debinski, W., Puri, R. K., Kreitman, R. J. and Pastan, I. (1993) J. Biol. Chem. **268**, 14065–14070
30 Spence, C., Nachman, M., Gately, M. K., Krietman, R. J., Pastan, I. and Bailon, P. (1993) Bioconjug. Chem. **4**, 63–68
31 Buchner, J., Pastan, I. and Brinkmann, U. (1992) Anal. Biochem. **205**, 263–270

32 Chaudhary, V. K., Gallo, M. G., FitzGerald, D. J. and Pastan, I. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 9491–9494
33 Tabas, I. and Kornfeld, S. (1979) J. Biol. Chem. **254**, 11655–11663
34 Kreitman, R. J., Chaudhary, V. K., Siegall, C. B., FitzGerald, D. J. and Pastan, I. (1992) Bioconjug. Chem. **3**, 58–62
35 Theuer, C. P., Buchner, J., FitzGerald, D. and Pastan, I. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 7774–7778
36 Semenza, J. C., Hardwick, K. G., Dean, N. and Pelham, H. R. B. (1990) Cell **61**, 1349–1357
37 Semenza, J. C. and Pelham, H. R. B. (1992) J. Mol. Biol. **224**, 1–5
38 Lewis, M. J. and Pelham, H. R. B. (1990) Nature (London) **348**, 162–163
39 Lewis, M. J. and Pelham, H. R. B. (1992) J. Mol. Biol.. **226**, 913–916
40 Haugejorden, S. M., Srinivasan M. and Green, M. (1991) J. Biol. Chem. **266**, 6015–6018

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