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Identification of the cellular receptor for anthrax toxin

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The tripartite toxin secreted by *Bacillus anthracis*, the causative agent of anthrax, helps the bacterium evade the immune system and can kill the host during a systemic infection. Two components of the toxin enzymatically modify substrates within the cytosol of mammalian cells: oedema factor (OF) is an adenylate cyclase that impairs host defences through a variety of mechanisms including inhibiting phagocytosis[1,2]; lethal factor (LF) is a zinc-dependent protease that cleaves mitogen-activated protein kinase kinase and causes lysis of macrophages[3–5]. Protective antigen (PA), the third component, binds to a cellular receptor and mediates delivery of the enzymatic components to the cytosol. Here we describe the cloning of the human PA receptor using a genetic complementation approach. The receptor, termed ATR (anthrax toxin receptor), is a type I membrane protein with an extracellular von Willebrand factor A domain that binds directly to PA. In addition, a soluble version of this domain can protect cells from the action of the toxin.

After binding to the cell-surface receptor, PA is cleaved into two fragments by a furin-like protease[6]. The amino-terminal fragment, PA_{20}, dissociates into the medium, and this allows the carboxy-terminal fragment, PA_{63}, to heptamerize and to bind LF and OF[7,8]. The resulting complexes of $[PA_{63}]_7$ with OF and/or LF are taken up into cells by receptor-mediated endocytosis and moved to a low-pH endosomal compartment[9]. There, the acidic environment induces a conformational change in $[PA_{63}]_7$ that allows it to insert into the membrane and form a pore[10–12]. This conversion promotes the translocation of bound OF and LF across the endosomal membrane to the cytosol.

Previous studies have indicated that the receptor to which PA binds is a ubiquitous protein expressed at moderately high levels on cell surfaces (for example, 10^4 and 3×10^4 receptors per cell on CHO-K1 cells and macrophage cell lines, respectively)[13,14]. To identify this receptor, we first generated a mutant cell line lacking receptor, so that the defect could be genetically complemented. ICR-191, a DNA alkylating agent that induces small deletions and frameshift mutations in genes[15], was used to introduce random mutations in the hypodiploid CHO-K1 cell line under conditions that led to about 90% cell death. The surviving mutagenized cells were then challenged with PA and LF_N–DTA, a fusion protein composed of the N-terminal 255 amino acids of LF linked to the catalytic A chain of diphtheria toxin[16]. This recombinant toxin can kill CHO-K1 cells (in contrast to LF and PA) and it exploits the same LF–PA–receptor interactions that are required for the binding and entry of the native LF and OF proteins. Ten single-cell colonies (designated as CHO-R1.1 to CHO-R1.10) that survived toxin treatment were isolated. In control experiments performed with non-mutagenized CHO-K1 cells, no toxin-resistant cell clones were detected. One of the mutagenized clones (CHO-R1.1) was chosen for further analysis.

CHO-R1.1 cells were fully susceptible to killing by diphtheria toxin (data not shown), thus ruling out the possibility that resistance to PA with LF_N–DTA was due to a defect in the pathway of DT action. To test directly whether CHO-R1.1 cells lacked the receptor, we performed flow cytometric analysis using an Oregon Green-conjugated form of PA (OGPA). CHO-R1.1 cells were significantly impaired in their ability to bind to OGPA compared with the parental cell line (Fig. 1a), suggesting that these mutagenized cells had lost expression of the putative PA receptor gene. Similar analysis of the other nine mutant CHO-R1 clones demonstrated that they were also defective in binding to OGPA (data not shown).

In an attempt to complement the PA-binding defect of CHO-R1.1 cells, the cells were transduced with a retrovirus-based complementary DNA library (Clontech) prepared from human HeLa cells that express the PA receptor (J.M., unpublished data). This cDNA library is contained in a murine leukemia virus (MLV) vector that is packaged into pseudotyped virus particles (MLV[VSV-G])

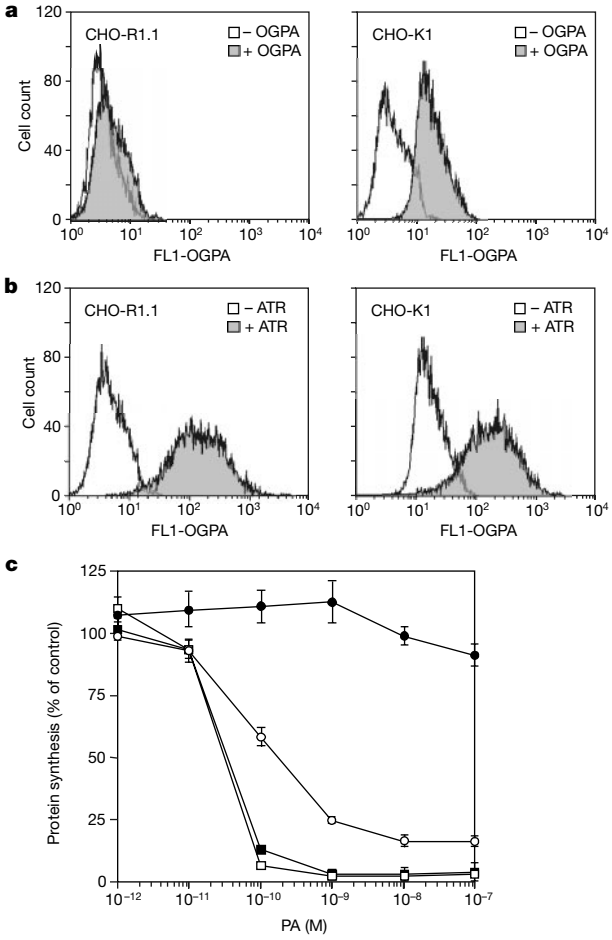

Figure 1 Mutant CHO-R1.1 cells display a decreased OGPA-binding phenotype that can be corrected by overexpression of the ATR cDNA. **a**, Mutant CHO-R1.1 and wild-type CHO-K1 cells were incubated with 40 nM OGPA for 2 h on ice, washed twice then analysed by flow cytometry. **b**, Mutant and wild-type CHO cells were transduced with an MLV vector encoding ATR and then stained with OGPA as above. **c**, Expression of ATR restores toxin sensitivity. CHO-R1.1 cells (filled circles), CHO-K1 cells (filled squares) and CHO-R1.1 and CHO-K1 cells transduced with the MLV vector encoding ATR (open circles and squares, respectively) were treated with 10^{-9} M LF_N–DTA and various concentrations of PA. About 70% of the transduced CHO-R1 cells expressed ATR as judged by OGPA straining. Medium containing 1 μCi ml^{-1} ^{3}H-leucine was then added to cells for 1 h, and the amount of ^{3}H-leucine incorporated into cellular proteins was determined by precipitation with trichloroacetic acid and liquid scintillation counting[16].

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containing the broad host-range G protein of vesicular stomatitis virus (VSV-G)[17]. Retrovirus-based cDNA libraries are useful for genetic complementation approaches because they can be used to deliver a limited number of stably expressed cDNA molecules per cell. These molecules can be rapidly re-isolated by polymerase chain reaction (PCR) amplification using MLV vector-specific oligonucleotide primers[18,19].

The transduced CHO-R1.1 cells were subjected to five rounds of flow cytometric sorting to isolate those that contained the cDNA clone of the putative PA receptor. Cells were sorted on the basis of their binding of OGPA in combination with an anti-PA polyclonal serum and an allophycocyanin (APC) conjugated secondary antibody. This led to the isolation of a cell population in which greater than 90% of the cells bound OGPA. This complemented cell population contained at least seven unique cDNA inserts that were obtained by the PCR amplification method described above. Each cDNA was gel purified, subcloned back into the parent pLIB vector and packaged into MLV(VSV-G) virions so that it could be tested for its ability to complement the PA-binding defect of CHO-R1.1 cells. One cDNA clone of about 1.5 kilobases (kb) (designated as *ATR*) restored PA binding to CHO-R1.1 cells (Fig. 1b). This clone also markedly enhanced the binding of PA to parental CHO-K1 cells (Fig. 1b). Furthermore, the *ATR* cDNA clone fully restored the sensitivity of CHO-R1.1 cells to the toxin LF_N–DTA with PA (Fig. 1c).

Sequencing of the *ATR* cDNA clone revealed a single long open reading frame, encoding a 368-amino-acid protein. The protein is predicted to have a signal peptide 27 amino acids long, an extracellular domain 293 amino acids long with three putative N-linked glycosylation sites, a putative transmembrane region 23 amino acids long, and a short cytoplasmic tail (Fig. 2). A BLAST search revealed that the first 364 amino acids of ATR are identical to a protein encoded by the human *TEM8* cDNA clone (GenBank accession number NM032208). TEM8 is upregulated in colorectal cancer endothelium, but the function of this protein has not been reported[20]. The C-terminal ends of ATR and the TEM8 protein then diverge, presumably as a consequence of alternative splicing, such that ATR has a cytoplasmic tail of only 25 amino acids whereas TEM8 is predicted to have a cytoplasmic tail 221 amino acids long (Fig. 2).

The most notable feature of ATR is the presence of an extracellular von Willebrand factor type A (VWA) domain, located between residues 44 and 216 (Fig. 2). VWA domains are present in the extracellular regions of a variety of cell surface proteins, including matrilins and integrins (designated as I domains). These domains are important for protein–protein interactions and constitute ligand-binding sites for integrins[21]. Ligand binding through I domains requires an intact MIDAS (metal ion-dependent adhesion site) motif[22], which seems to be conserved in ATR (Fig. 2). The cytoplasmic tail of ATR contains an acidic cluster (AC motif; EESEE) that is similar to a motif found in the cytoplasmic tail of furin that specifies basolateral sorting of this protease in polarized

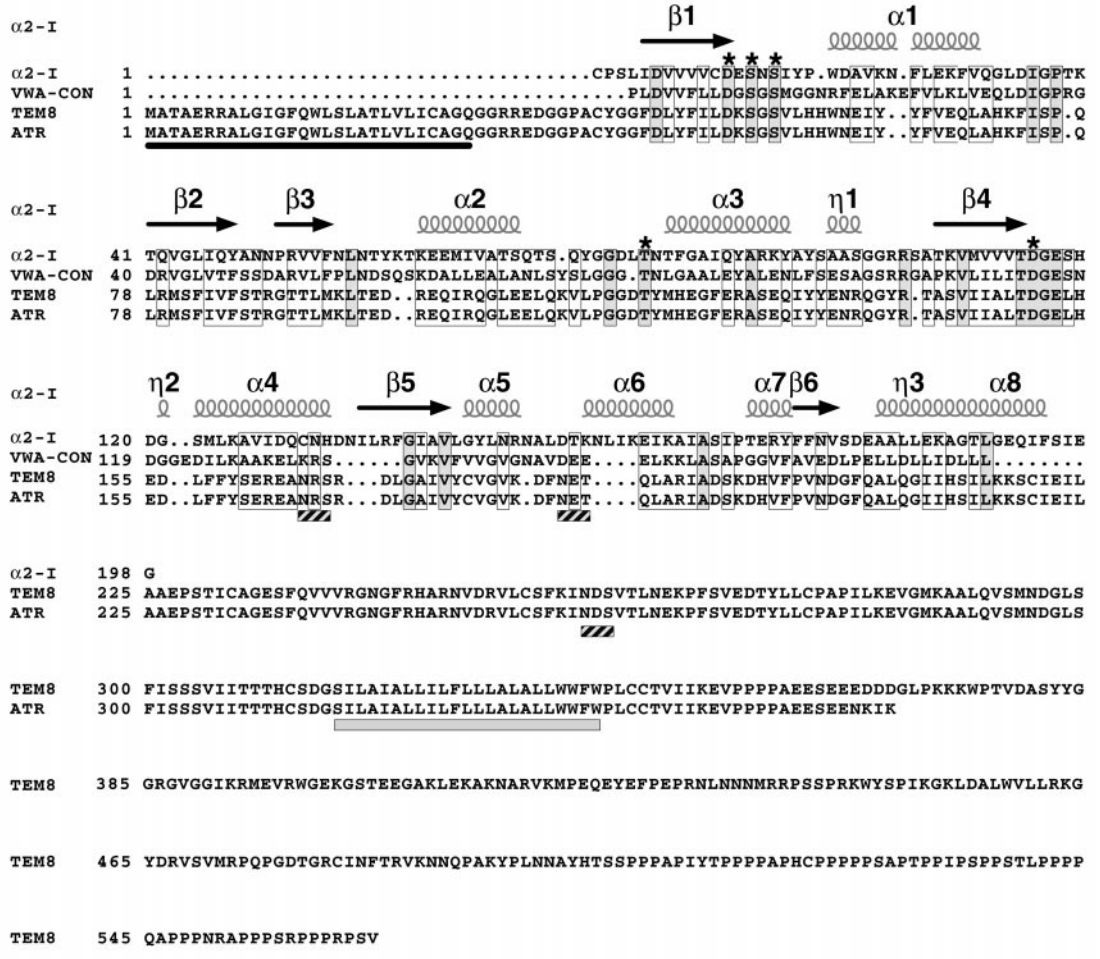

Figure 2 Sequence alignment of ATR with the I domain of integrin $\alpha 2$ ($\alpha 2$-I), the von Willebrand factor A domain consensus sequence (VWA-CON, generated from 210 sequences aligned by the National Center for Biotechnology Information), and TEM8. The secondary structural elements are based on the crystal structure of the $\alpha 2$-I domain[30]. Conserved amino acids are boxed and identical amino acids are indicated by shaded boxes. The putative signal sequence is underlined. The five residues that form the MIDAS motif are indicated with asterisks. The putative transmembrane domains of ATR and TEM8 are indicated by a shaded box. Potential N-linked glycosylation sites in ATR and TEM8 are indicated by hatched boxes. The alignment was made with the programs ClustalW and ESPript 1.9 (the Risler matrix was used with a global score of 0.7).

epithelial cells[23]. This may be significant because the PA receptor localizes to the basolateral surface of polarized epithelial cells[24] and we expect that the receptor and the protease needed to bind and activate PA would be colocalized to allow for efficient entry of anthrax toxins.

Given the likelihood that ATR is conserved among different species, it is of interest to note that the product of the mouse homologue of ATR/TEM8 (GenBank accession number AK013005) is highly related to the human clones, sharing greater than 98% sequence identity within the reported extracellular domain (data not shown). Furthermore, consistent with the observation that the anthrax toxin receptor is found in a variety of cell lines, *ATR* and/or *TEM8* is expressed in a number of different tissues including the central nervous system, heart, lung and lymphocytes (data not shown; and see NCBI UniGene cluster Hs.8966 at http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi ORG=Hs&CID=8966&OPT=text).

To confirm that PA binds directly to ATR, co-immunoprecipitations were performed with an extracellular fragment of ATR and either the wild-type or a mutant form of PA deficient in receptor binding. A fusion protein consisting of a hexahistidine tag, a T7 tag, and amino acids 41–227 of ATR (the I domain) was expressed and purified from *Escherichia coli* cells. When mixed with wild-type PA, this construct, T7–ATR_{41-227}, was precipitated with polyclonal anti-PA serum (Fig. 3a, lane 3). The interaction between PA and T7–ATR_{41-227} was impaired by the presence of EDTA (Fig. 3a, lane 5), demonstrating that the interaction requires divalent cations and suggesting that the MIDAS motif of ATR is critical for binding PA. In addition, a fusion protein consisting of glutathione S-transferase (GST) and the receptor-binding domain 4 (D4)[25,26] of PA (GST–D4) bound T7–ATR_{41-227}, whereas GST did not (Fig. 3b). PA-N682S, a mutant form of PA with residue Asn 682 replaced with Ser, is impaired in its ability to bind and intoxicate cells (Fig. 3c and d), and was unable to bind to T7–ATR_{41-227} (Fig. 3a, lane 4). These experiments demonstrate a direct and specific interaction between the VWA/I domain of ATR and the receptor-binding domain of PA.

Given this direct interaction, we reasoned that ATR_{41-227} might protect CHO-K1 cells from being killed by PA and LF_N–DTA. We tested this idea by mixing cells with an increasing amount of T7–ATR_{41-227} in the presence of a constant amount of PA and LF_N–DTA, and then measuring the subsequent effect on protein synthesis (Fig. 4). T7–ATR_{41-227} was an effective inhibitor of toxin action, inhibiting toxin activity by 50% and 100% at concentrations of 80 and 500 nM, respectively. T7–ATR_{41-227} did not, however, inhibit diphtheria toxin (data not shown).

We have identified a 368-amino-acid human protein, ATR, that contains a single extracellular VWA/I domain and serves as the cellular receptor for anthrax toxin. The clone that encodes this protein may be one of several alternatively spliced messenger RNA transcripts, including *TEM8*, that result from a primary mRNA. Because TEM8 also contains the extracellular VWA/I domain, which binds directly to PA, we predict that this clone may also function as a PA receptor. The identification of ATR now allows for a more detailed investigation of the mechanism of uptake by cells of anthrax toxin. Furthermore, that the soluble VWA/I domain of ATR inhibits toxin action, coupled with the use of the cloned receptor as a tool for identifying inhibitors of the PA–receptor interaction, holds promise for the development of new approaches for the treatment of anthrax.

Note added in proof: A further apparently full-length ATR/TEM8-related cDNA clone has been reported (GenBank accession code BC012074), which encodes a protein with yet another C-terminal end. □

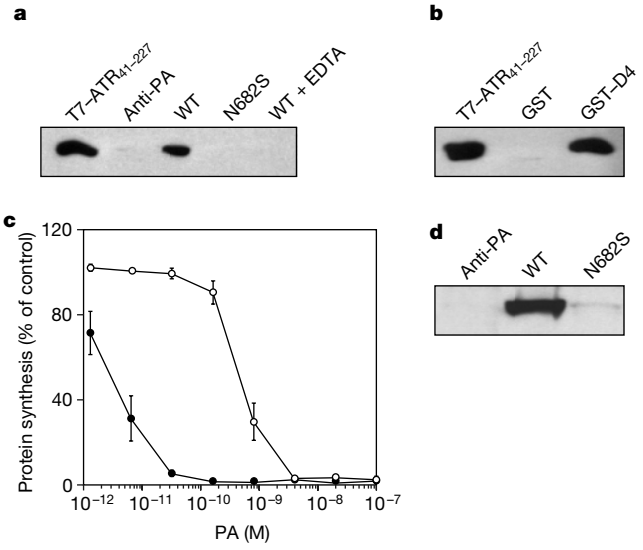

Figure 3 The VWA/I domain of ATR binds directly to PA. **a**, Wild-type PA (WT) or a receptor-binding mutant of PA (N682S) were mixed with T7–ATR_{41-227} on ice for 30 min in the presence or absence of 2 mM EDTA, as indicated. A polyclonal serum specific for PA and protein A sepharose were then added, the PA-associated proteins were precipitated, subjected to SDS–PAGE, transferred to nitrocellulose, and probed with anti-T7 antibody conjugated to horseradish peroxidase. **b**, GST or GST–D4 (GST fused to domain 4 of PA) coupled to glutathione sepharose (Pharmacia) was incubated with T7–ATR_{41-227} for 1 h at 4 °C and the samples were precipitated and analysed as described above. **c**, CHO-K1 cells were incubated with LF_N–DTA (10^{-9} M) and various concentrations of wild-type PA (filled circles) or PA-N682S mutant (open circles) and cell viability was determined as in Fig. 1c. **d**, CHO-K1 cells were incubated with 2×10^{-8} M trypsin-nicked PA (wild type or N682S) for 1 h. Cells were washed with PBS, resuspended in SDS sample buffer and run on a 4–20% SDS–PAGE gel. PA was visualized by western blotting. Lane 1 (**a**,**b**), T7–ATR_{41-227} loading control; lane 2 (**a**) and lane 1 (**d**) anti-PA serum control (no PA added).

Methods

Mutagenesis and characterization of CHO-K1 cells

About 5×10^7 CHO-K1 cells were treated at 37 °C for 7 h with medium containing 10 μg ml^{-1} ICR-191 (Sigma) then washed twice. After 4 d, surviving cells were replated and incubated for 3 d with medium containing 8 μg ml^{-1} PA and 10 ng ml^{-1} LF_N–DTA. Surviving single-cell clones were isolated 14 d later. CHO-R1.1 cells were assayed for their sensitivity to DT intoxication by measuring incorporation of ^{3}H-leucine into cellular proteins after exposure to the toxin[16]. Flow cytometry analysis was performed after incubating cells at 4 °C for 2 h in medium containing 40–80 nM OGPA (PA-K563C

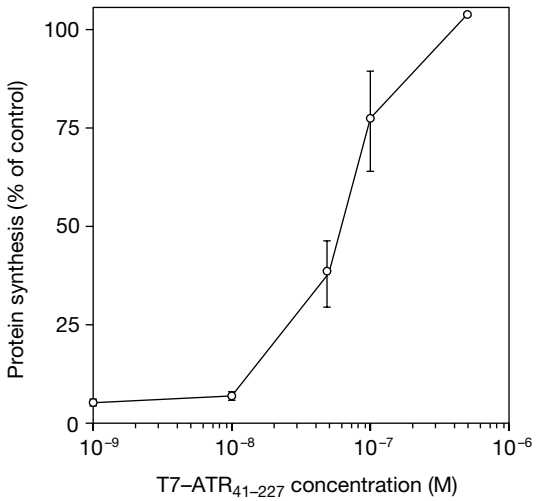

Figure 4 T7–ATR_{41-227} protects cells from killing by PA with LF_N–DTA. CHO-K1 cells were incubated at 37 °C for 4 h with 10^{-10} M PA, 2.5×10^{-11} M LF_N–DTA and increasing amounts of T7 ATR_{41-227}. Toxin sensitivity was determined by measuring the inhibition of protein synthesis as determined in Fig. 1c.

coupled to Oregon Green maleimide (Molecular Probes)). The cells were then washed twice with medium and analysed with a Becton Dickinson FACSCalibur flow cytometer.

cDNA complementation

About 5×10^5 CHO-R1.1 cells were transduced with $\sim10^7$ infectious units (complexity of library = 2×10^6 independent clones) of the pLIB-based cDNA library (Clontech) produced in the 293GPG packaging cell line[27]. Three days later, cells were incubated with medium containing 80 nM OGPA and the top 0.1% of fluorescent cells were then isolated by sorting using a Becton Dickinson FACSVantage SE instrument. These cells were expanded and subjected to four additional rounds of sorting using OGPA as above, as well as a 1:500 dilution of a rabbit anti-PA polyclonal serum along with a 1:500 dilution of an APC-conjugated secondary antibody (Molecular Probes). OGPA single positive (round 2) or OGPA/APC double positive (rounds 3–5) cells were recovered (the top 20%, 1%, 5% and 50% of fluorescent cells for rounds 2, 3, 4 and 5, respectively) and expanded after each round of sorting. The cDNA inserts contained within these cells were recovered by PCR amplification of genomic DNA samples with oligonucleotide primers specific for the MLV vector according to the manufacturer's instructions (Clontech). Each cDNA was sub-cloned between the *Not*I and *Sal*I restriction enzyme sites of pLIB and the resulting plasmids were cotransfected into 293 cells with MLV gag/pol and VSV-G expression plasmids pMD.old.gagpol and pMD.G[28]. Resulting pseudotyped virus particles were used to infect CHO-R1.1 and CHO-K1 cells followed by OGPA staining and FACS analysis as above. All sequencing was performed by the McArdle Laboratory macromolecular core.

Cloning and expression of T7–ATR_{41-227}

A DNA fragment encoding amino acids 41–227 of ATR was cloned into the *Bam*H1 and *Eco*R1 sites of pET28A (Novagen) to generate pET28A-ATR_{41-227}. BL21 (DE3) cells (Stratagene) containing pET28A-ATR_{41-227} were grown at 37 °C to an absorbence at 600 nm (A_{600}) of 0.6, induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h and collected by centrifugation. The cells from 1.5 l of culture were resuspended in 25 ml of 50 mM Tris-HCl buffer at pH 8.0, 2 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride, and were passed through a French press. We added 1 mg of DNase I (Roche) to the cell lysate, which we then sonicated for 1 min and centrifuged at 21,000*g* for 20 min. The pellet was resuspended in 25 ml of 50 mM Tris-HCl at pH 8.0 and 2 mM DTT, and centrifuged at 21,000*g* for 20 min. This wash step was repeated once. T7–ATR_{41-227} was solubilized and folded essentially as described previously[29].

Isolation of PA-N682S

The DNA encoding domain 4 of PA was mutagenized by error-prone PCR[31]. Clones were expressed in *E. coli*, and lysates derived from these clones were added to CHO-K1 cells in combination with LF_N–DTA. Clones corresponding to lysates that did not kill CHO-K1 cells were sequenced and the N682S mutant clone was further characterized here.

Co-immunoprecipitation of PA and T7–ATR_{41-227}

A mixture of 5 μg PA (wild type or N682S) and 2 μg T7–ATR_{41-227} (in 20 mM Tris-HCl at pH 8.0, 150 mM NaCl and 0.1 mg ml^{-1} bovine serum albumin) was incubated on ice for 30 min in the presence or absence of 2 mM EDTA. Anti-PA polyclonal serum (10 μl) was added to this solution and incubated on ice for an additional 1 h. Protein A agarose (Santa Cruz Biotechnology) was added and the solution was rotated at 4 °C for 1 h, then washed four times with 20 mM Tris-HCl at pH 8.0 and 150 mM NaCl. About one-third of the mixture was subjected to SDS–PAGE, transferred to nitrocellulose and probed with anti-T7 antibody conjugated to horseradish peroxidase (Novagen).

GST–D4 pull-down assay

DNA encoding amino acids 595–735 of PA (domain 4) was cloned into pGEX-4T-1 (Pharmacia Biotechnology). GST–D4 was coupled to glutathione sepharose at 4 mg ml^{-1} GST–D4 according to the manufacturer's instructions (Pharmacia Biotechnology). GST or GST–D4 coupled to glutathione sepharose was mixed with 2 μg of T7–ATR_{41-227} and 250 μg of *E. coli* extract in 250 μl for 1 h at 4 °C. The beads were washed four times with 20 mM Tris-HCl at pH 8.0 and 150 mM NaCl. One-half of the suspension was subjected to SDS–PAGE, transferred to nitrocellulose, and probed with anti-T7 antibody coupled to horseradish peroxidase.

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Competing interests statement

The authors declare competing financial interests: see the website (http://www.nature.com) for details.

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