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(54) **ANTIGENE 791GP72 ASSOCIE A UNE TUMEUR**

(54) **TUMOR ASSOCIATED ANTIGEN 791TGP72**

(57) Vaccins contre le cancer comprenant un polypeptide de la famille CD55, ou des fragments ou des dérivés de polypeptides de la famille CD55. Un polypeptide préféré est l'antigène 791gp72. L'invention concerne également des antigènes 791gp72 isolés et purifiés.

(57) Cancer vaccines comprising a polypeptide of the CD55 family, or fragments or derivatives of polypeptides of the CD55 family. A preferred polypeptide is the 791Tgp72 antigen. Also provided are isolated and purified 791Tgp72 antigens.

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(54) Title: TUMOR ASSOCIATED ANTIGEN 791Tgp72 (57) Abstract Cancer vaccines comprising a polypeptide of the CD55 family, or fragments or derivatives of polypeptides of the CD55 family. A preferred polypeptide is the 791Tgp72 antigen. Also provided are isolated and purified 791Tgp72 antigens.		

Tumour Associated Antigen 791Tgp72Field of the Invention

5 The present invention relates to tumour associated antigen 791Tgp72 and a method for its isolation, and to the use of 791Tgp72 and/or CD55 and related substances in methods of medical treatment, in particular as cancer vaccines.

10 Background of the Invention

A mouse monoclonal antibody 791T/36 was raised against the osteosarcoma cell line 791T (Embleton et al, 1981). A cell line expressing this antibody is deposited with the ATCC under accession number HB9173.

15 Immunoprecipitation studies showed that 791T/36 recognised a membrane glycoprotein of molecular weight 72,000 (Price et al, 1984). A similar antigen can also be precipitated from activated human T lymphocytes. Extensive studies have shown that 791T/36 binds to the

20 majority of osteosarcomas and also to colorectal, gastric and ovarian tumours (Durrant et al, 1986; Durrant et al, 1989; Durrant et al, 1989). The tumour specificity of 791Tgp72 was also emphasised by extensive clinical imaging studies with radiolabelled 791T/36 in the

25 detection of primary and metastatic colorectal cancer, osteosarcoma, breast and ovarian cancer. The antibody was also linked to ricin A chain and showed good killing of tumour cells expressing the 791Tgp72 antigen. A phase I clinical study showed that the dose limiting toxicity

30 was due to vascular leak syndrome and neurological toxicity of the ricin and was unrelated to antibody binding.

During the course of the clinical imaging and toxin targeting studies with 791T/36, it became clear that a

limitation was the induction of human anti-mouse antibody responses (HAMA) (Durrant et al, 1989) which could limit the effectiveness of subsequent therapy with this monoclonal antibody. A large component of this HAMA response was directed at the idiotype of 791T/36. Most patients made a very strong anti-idiotypic response suggesting that a pre-existing helper T-cell response to tumour expressed 791Tgp72 antigen allowed preferential help for an anti-idiotypic response. Indeed a patient who had already survived 3 years with metastatic colon cancer received radiolabelled 791T/36 for tumour imaging. He made a very strong idiotypic response which resulted in anaphylactic shock suggesting that the pre-existing helper response to the 791Tgp72 may have been stabilising his disease and had been boosted with the injection of 791T/36. He recovered and lived a further 4 years finally succumbing to bone metastases. A human monoclonal anti-idiotypic antibody which bound to the antigen combining site of 791T/36 was produced from this patient (Austin et al, 1989 and WO90/04415). Similarly immunisation of mice with 791T/36 linked to ricin induced a strong anti-idiotypic response and a mouse monoclonal anti-idiotypic antibody to 791T/36 was produced.

Clinical and laboratory studies with the human anti-idiotypic antibody have shown that it is an excellent immunogen for stimulating anti-tumour T-cell mediated immunity. 105AD7 can prime delayed hypersensitivity responses in rats and mice to human tumour cells expressing 791Tgp72 antigen. No toxicity related to anti-idiotypic immunisation has been observed in any of the 164 patients entered into phase I/II clinical trials with 105AD7. Patients in the phase I study showed T-cell proliferation responses to both the 105AD7 immunogen and

also to the target antigen 791Tgp72 which correlated with survival. The lack of toxicity and excellent immune responses has enabled us to undertake a trial in primary colorectal cancer patients where evidence of autologous anti-tumour cytotoxicity was observed in patients immunised with 105AD7 prior to surgery. Single CTL epitope vaccines may not be very effective as some tumour cells lack the target antigen. This is less of a problem when stimulating helper T-cell responses due to different effector mechanisms. Antigen stimulation and homing occur by a similar mechanism to CTL. However, once at the tumour site, helper T-cells release cytokines which initiate a cascade of inflammatory events resulting in recruitment of effector cells which can kill tumour cells independent of their antigen status. This kind of infiltration profile has been seen in the tumours of patients following 105AD7 immunisation. CD4 and CD8 T-cells and natural killer cells were elevated in immunised patients compared to unimmunised. Furthermore, immunised patients had enhanced natural killer cell activity, which is of great significance as colorectal tumours often lose expression of MHC molecules resulting in susceptibility to NK killing.

25 Summary of the Invention

Previous attempts to purify and identify the 791Tgp72 antigen using both immunoprecipitation and affinity chromatography failed due to poor yields and the conformational dependence of 791T/36 for antigen binding. A modified method of affinity purification of 791Tgp72 has now been developed which has led to the isolation of this antigen for the first time. Biotinylation of cell membranes has allowed us to optimise the purification

protocol, enabling efficient tracing of purified fractions. The use of the mild detergent octyl-glucoside and the introduction of an ultracentrifugation step has enhanced the purification 50-100 fold. The affinity chromatography has significantly been improved by covalently coupling the capturing antibody (791T/36) to protein-A sepharose. We have purified over 100 μ g of the antigen and N-terminal sequencing has identified the molecule as being a member of the CD55/DAF family.

Further sequencing has revealed that the coding region of 791Tgp72 cDNA is the same as that for a known CD55 protein (herein known as "CD55"). Hence the amino acid sequences of 791Tgp72 and CD55 are also identical. There are, however, differences between the 791Tgp72 and CD55 proteins, for example in the glycosylation pattern of the molecules.

Accordingly, in a first aspect, the present invention provides isolated and purified 791Tgp72 antigen.

In a further aspect, the present invention provides isolated and purified 791Tgp72 antigen as obtainable by:

- (a) solubilising 791T cells in lysis buffer including 1% octyl-B-glucoside, pH 8.5 for 1 hour at 4°C;
- (b) centrifuging the lysate at 13000 rpm x 10 min following 100,000 g x 30 min;
- (c) adding the cleared lysate to Protein A sepharose coupled to 791T/36 affinity column;
- (d) cycling the supernatant over the column at 0.3-0.4 ml/min;
- (e) washing the column with 20ml 20 mM Tris-HCl pH 8.0 containing 0.3 M NaCl and 0.1% NP-40; and,
- (f) eluting 791Tgp72 from the column in 5 column volumes of diethylamine pH 11.5 containing 0.5% NP-40 and

neutralising the eluate with 1M Tris.

In a further aspect, the present invention provides a pharmaceutical composition comprising 791Tgp72 in combination with a pharmaceutically acceptable carrier.

5 In a further aspect, the present invention provides 791Tgp72 for use in a method of medical treatment.

In a further aspect, the present invention provides a method for isolating 791Tgp72 antigen from cells expressing 791Tgp72, the method including the steps of:

10 solubilising the cells with lysis buffer including octyl-glucoside; and

treating the lysate using ultracentrifugation.

The inventors found that these steps surprisingly helped to enhance the purification of the antigen 50-100 fold.

15 The isolation and characterisation of 791Tgp72 carried out for the first time here identified this antigen as a member of the CD55 or decay accelerating factor (DAF) family. Thus, the use of 791Tgp72 as a cancer vaccine proposed herein can be extended to other CD55 polypeptides, a variety of forms of which have been isolated in the prior art, and to fragments and derivatives of these molecules. Likewise, the use of nucleic acid sequences encoding 791Tgp72 or its fragments and derivatives can be extended to nucleic acid sequences encoding other CD55 family members, and their fragments and derivatives.

25 Accordingly, in a further aspect, the present invention provides a cancer vaccine comprising 791Tgp72 antigen or a polypeptide of the CD55 family, or a fragment or derivative of 791Tgp72 or of a polypeptide of the CD55 family, wherein the vaccine is capable of inducing an immune response in a patient. The response

may be one or more of a T-helper cell response, a cytotoxic T-cell response, an NK cell response and/or an immune response.

5 In a further aspect, the present invention provides a cancer vaccine comprising nucleic acid encoding 791Tgp72 and/or a polypeptide of the CD55 family, or a fragment or derivative of T791Tgp72 or of a polypeptide of the CD55 family, wherein the vaccine is capable of inducing an immune response in a patient. Again, the
10 response may be one or more of a T-helper cell response, a cytotoxic T-cell response, an NK cell response and/or an immune response.

In a further aspect, the present invention provides the use of 791Tgp72 antigen or a polypeptide of the CD55
15 family, or a fragment or derivative of T791Tgp72 or of a polypeptide of the CD55 family, in the preparation of a medicament for the treatment of cancer.

In a further aspect, the present invention provides the use of nucleic acid encoding 791Tgp72 antigen or a
20 polypeptide of the CD55 family, or a fragment or derivative of T791Tgp72 or of a polypeptide of the CD55 family, in the preparation of a medicament for the treatment of cancer.

In a further aspect, the present invention provides
25 a method of treating a patient having cancer, the method comprising administering to the patient a therapeutically effective amount of one of the above cancer vaccines.

The use of 791Tgp72 to stimulate a T-cell response to cancer cells bearing 791Tgp72 is unexpected, as it is
30 thought that the cancer cells have evolved to express these antigens to protect them from complement mediated attack. Thus, it is surprising that this defence mechanism of the tumour cells provides a way of

selectively targeting a T-cell response to cancer cells expressing high levels of these antigens. By way of example, the vaccine can be used to treat colorectal cancer, osteosarcoma, breast and ovarian cancer, all of which are associated with 791Tgp72 overexpression.

While 791Tgp72 antigen and CD55 are known to be over-expressed on a wide range of solid tumours, they are also expressed on normal red blood cells, leukocytes, endothelial cells and surface epithelial cells. However, the T-cell response induced by employing a vaccine based on these polypeptides should be capable of discriminating between the low level of expression on normal cells and the high levels on tumour cells. This is based on the observation that the binding of 791T/36 to tumour cells shows higher affinity than binding to red blood cells from experiments in which the passage of red blood cells through tumours resulted in transfer of the monoclonal antibody to the tumour cells. Thus, this suggests that the T-cell response will be targeted to tumours and immune clearance is avoided.

Clinical studies with the human monoclonal antibody 105AD7 which mimics the colorectal tumour associated antigen 791Tgp72 have shown that immunised patients show a range of anti-tumour T-cell responses as exemplified by antigen specific proliferation responses, enhanced IL-2 production, induction of CD45RO cells, infiltration of CD4, CD8 and CD56 cells within the tumours of immunised patients, enhanced natural killer activity and autologous tumour killing which was unrelated to NK killing. As the 105AD7 antibody vaccine has now been given to 164 patients with no associated toxicity, vaccines based on 791Tgp72 or CD55 may share this property.

Results below supporting the use of polypeptides of

the CD55 family in an analogous manner to 791Tgp72 include:

- (a) Sequence identity of 791Tgp72 and CD55.
- (b) Monoclonal antibodies specific to CD55 bind to
5 purified 791Tgp72 antigen.
- (c) 791T/36 binds to cells transfected with CD55.
- (d) 791T/36 binds to cells transfected with
CD55/C46 chimeric constructs which contain CD55 SUSHI
domain 2.
- 10 (e) 791T/36 and monoclonal antibodies specific to
CD55 immunoprecipitate two proteins of 72 and 66 kDa from
the 791T osteosarcoma cell line. However, the yield of
the dimer is far greater with 791T/36 than with the anti-
CD55 monoclonal antibodies.
- 15 (f) 791T/36 and monoclonal antibodies specific to
CD55 immunoprecipitate a single band of 72kDa from normal
red blood cells.
- (g) 791T/36 recognises an epitope on 791Tgp72 as
expressed by osteosarcoma cells, but binds weakly to red
20 blood cells. In contrast, BRIC 216 (Blood Group
Reference Laboratory, Bristol, UK), a monoclonal antibody
which recognises CD55 as expressed by red blood cell,
binds less well to osteosarcoma tumour cell lines as
compared to 791T/36.
- 25 (h) 791Tgp72 is a GPI linked protein which is
released by phospholipase C treatment.
- (i) Radiolabelled 791T/36 localised within the
ovarian and colorectal tumours and showed no detectable
binding to red or white blood cells.
- 30 (j) 105AD7, an anti-idiotypic antibody which mimics
791Tgp72, has amino acid homology with the SCR2 domain
(also known as SUSHI domain 2) of CD55.
- (k) 730, an anti-idiotypic antibody which mimics

791Tgp72, has amino acid homology with the SCR2 domain of CD55.

(1) Ab3 responses induced by either the human or the mouse anti-idiotypic may bind to CD55 on activated T-cells and enhance proliferation.

Furthermore, analysis of the amino acid sequence of CD55/791Tgp72 indicates that it may contain other T cell epitopes, which are distinct from the epitopes mimicked by 105AD7 and 730 anti-idiotypic antibodies. This suggests that vaccines comprising these other epitopes may induce immune responses in a broader range of patients than vaccines prepared from the anti-idiotypic antibodies.

The present invention will now be described by way of example and not limitation, with reference to the accompanying figures.

Brief Description of the Figures

Figure 1 shows the detection of biotinylated proteins following SDS-PAGE and Western blotting. 791T cells were biotinylated and anti-DAF antibody (791T/36) or control antibody (1143/B7) was added either before or after solubilisation with 1% NP-40. The effect of crosslinking reagent (DTSSP) was assessed in the precipitation. X = Solubilisation after monoclonal antibody incubation with cells for 1 hour. O = Solubilisation of cells prior to addition of antibody.

Figure 2 shows SDS-PAGE analysis of 791T/gp72 immunoprecipitates from cell surface biotinylated 791T cells. The samples were detected by the ECL reagent on Western blotted gels. The gel represents the effects of varying detergents and centrifugation protocols on sample purification.

Figure 3 shows SDS-PAGE and silver staining of samples during protein purification. Lane 1, protein marker; (2) cell lysate; (3) unbound sample after passing the column; (4-7) samples from four consecutive 5 ml column washes; (8) concentrated washings; (9) samples of column eluate; and (10) concentrated column eluate. Each of the samples run on the gel was 25 μ l volume. The washing and elution volumes were 5 ml.

Figure 4 shows analysis of affinity purified 791T/gp72 by 7% SDS-PAGE and detected by silver staining. Lanes 1-5, 25 μ l samples from consecutive 1.2 ml diethylamine eluates from the Protein-A affinity column. Lanes 6-10, varying concentrations of purified BSA.

Figure 5 shows immunoprecipitation of cell surface biotinylated 791T cells by antibodies to DAF (110, BRIC 216) anti 791T/gp72 (791T/36) and anti EGF receptor monoclonal antibody (340). Experiments were carried out with the same amount of antibody, analysed by SDS-PAGE and western blotting and detecting using the ECL system. Lane 1 represents purified 791T/gp72. Lanes 4-7 represent precipitation with monoclonal antibodies 110, BRIC 216, 791T/36 and 340 respectively. Significantly more antigen is precipitated by 791T/36 compared to the anti-DAF antibodies.

Figure 6 shows binding of anti-DAF antibodies (110, BRIC 216) and 791T/36 to affinity purified 791Tgp72 antigen and to PI-PLC released antigen from 791T cells.

Figure 7 shows sandwich ELISA to determine if 791T/36 and the anti-DAF antibodies were binding distinct domains. Plates were coated with 791T/36, control antibody 708 (IgG2b) or anti-DAF antibodies; 220 (SUSHI domain 1), 110 (SUSHI domain 2), BRIC 216 (SUSHI domain 3). Binding of 791Tgp72 was detected with FITC-791T/36.

Figure 8 shows non-reducing SDS-PAGE of proteins from erythrocyte and 791T cell membranes. Gels were Western blotted, cut into lanes, probed with appropriate antibody and developed using the ECL system.

5 Figure 9 shows clustal alignment of CD55 and cloned products from 791T cells. 5/P5 represents 5 clones sequenced from the primer sequence P5. RC of B/C DAF represents 5 clones sequenced from the primers B DAF 3' and c DAF 3'. CDAF.seq is the full length sequence of
10 CD55 taken from GENBANK.

Figure 10 shows the full length amino acid sequence of CD55 and the cDNA sequence which encodes it.

Figure 11 shows the full length cDNA sequence of 791Tgp72 and the deduced amino acid sequence. 791Tgp72
15 has been found to have an identical amino acid sequence to CD55 and to be encoded by cDNA which is identical over the entire coding region to the cDNA encoding CD55, though differences exist in the 5' and 3' non-coding regions. These differences may be attributable to the
20 use of different primers.

Detailed Description

CD55, DAF and 791Tgp72 Polypeptides

"791Tgp72" refers to the tumour associated antigen
25 isolated in the work described herein from 791T cells that is bound by antibody 791T/36 (Embleton et al, 1981). This antigen is a member of the CD55 family, and has a high degree of amino acid homology with this known polypeptide. However, there are other differences
30 between 791Tgp72 and other CD55 polypeptides, for example in the glycosylation pattern of the molecules. Further, different RNAs encoding 791Tgp72 antigen have been observed in the work described below and these may encode polypeptides having variations in amino acid sequence as
35 compared to CD55.

"CD55" refers particularly to the polypeptide having the sequence shown in figure 10. CD55 is also known as

decay accelerating factor (DAF) and a variety of alternative forms of the polypeptide are known.

As used herein, "CD55 family" includes sequences which share substantial homology with CD55, such as the
5 aforementioned alternative forms of the polypeptide (e.g. the previously identified CD55-A, CD55-B and CD55-U2), and which are capable of inducing in a patient an immune response against CD55 and/or 791Tgp72 as expressed on cancer cells. Preferably the degree of homology between
10 CD55 and another protein of the CD55 family will be at least 60%, more preferably 70%, further preferably 80%, even more preferably 90%, or most preferably 95%.

CD55 was first purified by Nicholson-Weller et al from guinea pig and human erythrocytes (see Nicholson-
15 Weller et al, 1981, 1982). Purified CD55 is a single chain glycoprotein with an Mr of 60,000 (guinea pig) or 70,000 (human) on SDS-PAGE. CD55 is initially synthesised as a precursor of 46 kDa, which gives rise to the mature CD55 on the cell surface with an MW of 70,000
20 to 80,000 due to heterogeneity in glycosylation. The structure of CD55 has been elucidated by a combination of biochemical studies and by the molecular cloning of cDNA. The cDNA for human CD55 encodes a 34-amino acid signal peptide followed by a 347-amino acid sequence of the
25 protein. The amino terminus of the protein consists of four CCPR domains (also known as SUSHI or SCR domains). CD55 is anchored through covalent attachment to a GPI anchor.

As shown herein, antigen 791Tgp72 has an identical
30 amino acid sequence to that of CD55 as shown in Fig 10. The results described below suggest that CD55 and 791Tgp72, and fragments and derivatives thereof, can be used as cancer vaccines, to induce immune responses such

as anti-tumour T-cell responses as exemplified by antigen specific proliferation responses, T-helper cell responses, cytotoxic T-cell responses, enhanced IL-2 production, induction of CD45RO cells, infiltration of CD4, CD8 and CD56 cells within the tumours of immunised patients, enhanced natural killer activity and/or autologous tumour killing which was unrelated to NK killing. Further, the peptides may act to raise CTL antibodies that neutralise CD55 and allow complement mediated lysis to take place.

Accordingly, the invention further includes the use of "fragments" or "derivatives" of either 791Tgp72 or other polypeptides of the CD55 family, which are less than the full length polypeptides, but which are capable of inducing an anti-tumour immune (especially T-cell) response as assessed by one or more of the indicators above. A preferred group of fragments are those which include all or part of the SUSHI2 domain of CD55 that stretches between amino acids 97-159 of full length CD55.

A "fragment" of a 791Tgp72 or of a polypeptide of the CD55 family means a stretch of amino acid residues of at least about five to seven contiguous amino acids, often at least about seven to nine contiguous amino acids, typically at least about nine to 13 contiguous amino acids, more preferably, at least about 20 to 30 or more contiguous amino acids, and most preferably at least about 30 to 40 or more consecutive amino acids.

A "derivative" of 791Tgp72 or of a polypeptide of the CD55 family, or of a fragment of 791Tgp72 or CD55 family polypeptide, means a polypeptide modified by varying the amino acid sequence of the protein, e.g. by manipulation of the nucleic acid encoding the protein or by altering the protein itself. Such derivatives of the

natural amino acid sequence may involve insertion, addition, deletion and/or substitution of one or more amino acids, while providing a peptide capable of inducing an anti-tumour T-cell response.

5 Preferably such derivatives involve the insertion, addition, deletion and/or substitution of 25 or fewer amino acids, more preferably of 15 or fewer, even more preferably of 10 or fewer, more preferably still of 5 or fewer and most preferably of 1 or 2 amino acids only.

10 The invention also includes derivatives of the above peptides, including the peptide linked to a coupling partner, e.g. an effector molecule, a label, a drug, a toxin and/or a carrier or transport molecule. Techniques for coupling the peptides of the invention to both
15 peptidyl and non-peptidyl coupling partners are well known in the art. In one embodiment, the carrier molecule is a 16 amino acid peptide derived from the homeodomain of *Antennapedia* (e.g. as sold under the name "Penetratin"), which can be coupled to a peptide via a
20 terminal Cys residue. The "Penetratin" molecule and its properties are described in WO 91/18981.

 Peptides may be generated wholly or partly by chemical synthesis. The compounds of the present invention can be readily prepared according to well-
25 established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, *Solid Phase Peptide Synthesis*, 2nd edition, Pierce Chemical Company, Rockford, Illinois
30 (1984), in M. Bodanzsky and A. Bodanzsky, *The Practice of Peptide Synthesis*, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be prepared in solution,

by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

Another convenient way of producing a peptidyl molecule according to the present invention (peptide or polypeptide) is to express nucleic acid encoding it, by use of nucleic acid in an expression system.

Accordingly the present invention also provides in various aspects nucleic acid encoding the polypeptides and peptides of the invention.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human genome, except possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA. Where nucleic acid according to the invention includes RNA, reference to the sequence shown should be construed as reference to the RNA equivalent, with U substituted for T.

Nucleic acid sequences encoding a polypeptide or peptide in accordance with the present invention can be readily prepared by the skilled person using the information and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, and

Ausubel et al, Short Protocols in Molecular Biology, John Wiley and Sons, 1992), given the nucleic acid sequence and clones available. These techniques include (i) the use of the polymerase chain reaction (PCR) to amplify
5 samples of such nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparing cDNA sequences. DNA encoding 791Tgp72 or CD55 fragments may be generated and used in any suitable way known to those of skill in the art, including by taking encoding DNA,
10 identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another
15 recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Modifications to the sequences can be made, e.g. using site directed mutagenesis, to lead to the expression of modified peptide or to take account of codon preference in the
20 host cells used to express the nucleic acid.

In order to obtain expression of the nucleic acid sequences, the sequences can be incorporated in a vector having one or more control sequences operably linked to the nucleic acid to control its expression. The vectors
25 may include other sequences such as promoters or enhancers to drive the expression of the inserted nucleic acid, nucleic acid sequences so that the polypeptide or peptide is produced as a fusion and/or nucleic acid encoding secretion signals so that the polypeptide
30 produced in the host cell is secreted from the cell. Polypeptide can then be obtained by transforming the vectors into host cells in which the vector is functional, culturing the host cells so that the

polypeptide is produced and recovering the polypeptide from the host cells or the surrounding medium.

Prokaryotic and eukaryotic cells are used for this purpose in the art, including strains of E. coli, yeast,
5 and eukaryotic cells such as COS or CHO cells.

Thus, the present invention also encompasses a method of making a polypeptide or peptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide or peptide (generally nucleic
10 acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides and peptides may also be expressed in in vitro systems, such
15 as reticulocyte lysate.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems.
20 Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is E. coli.

25 Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral
30 e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and

protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Thus, a further aspect of the present invention provides a host cell containing heterologous nucleic acid as disclosed herein.

The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. As an alternative, direct injection of the nucleic acid could be employed.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones

containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by
5 culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide (or peptide) is produced. If the polypeptide
10 is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide or peptide may be isolated and/or purified from the host cell and/or culture medium, as the case may
15 be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see
20 below).

Pharmaceutical Formulations

The polypeptides, derivatives and fragments of the invention can be formulated in pharmaceutical
25 compositions, and especially as vaccine compositions. These compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials
30 should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or

subcutaneous, nasal, intramuscular, intraperitoneal routes. The formulation is preferably liquid, and is ordinarily a physiologic salt solution containing non-phosphate buffer at pH 6.8-7.6, or may be lyophilized powder.

The compositions comprising or for the delivery of the 791Tgp72 and/or CD55 polypeptides are preferably administered to an individual in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. The vaccines of the invention are particularly relevant to the treatment of existing cancer and in the prevention of the reoccurrence of cancer after initial treatment or surgery. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A. (ed), 1980.

791Tgp72 antigen and/or polypeptides of the CD55 family, and/or their fragments and/or derivatives are prepared for administration by mixing them at the desired degree of purity with adjuvants or physiologically acceptable carriers, i.e. carriers which are non toxic to recipients at the dosages and concentrations employed.

Adjuvants and carriers are substances that in themselves share no immune epitopes with the target antigen, but which stimulate the immune response to the target antigen. Ordinarily, this will entail combining active ingredient with buffers, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose or dextrans, chelating agents such as EDTA, and other excipients. Freund's adjuvant (a mineral oil emulsion) has commonly been used for this purpose, as have a variety of toxic microbial substances such as mycobacterial extracts and cytokines such as tumour necrosis factor and interferon gamma. Other adjuvants for vaccination are disclosed in EP-A-0745388, WO97/01330 and EP-A-0781559. Carriers can also act as adjuvants, but are generally distinguished from adjuvants in that carriers comprise water insoluble macromolecular particulate structures which aggregate the antigen, typical carriers include aluminum hydroxide, latex particles, bentonite and liposomes.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated. Other cancer treatments include the 105AD7 antibody mentioned above, other chemotherapeutic agents, other radiotherapy techniques or other cancer vaccines known in the art. One particular application of the compositions of the invention are as an adjunct to surgery, i.e. to help to reduce the risk of cancer reoccurring after a tumour is removed.

It is envisioned that injections (intramuscular or subcutaneous) will be the primary route for therapeutic administration of the vaccines of this invention, intravenous delivery, or delivery through catheter or

other surgical tubing is also used. Liquid formulations may be utilized after reconstitution from power formulations.

The polypeptide may also be administered via
5 microspheres, liposomes, other microparticulate delivery systems or sustained release formulations placed in certain tissues including blood. Suitable examples of sustained release carriers include semipermeable polymer matrices in the form of shaped articles, e.g.
10 suppositories, or microcapsules. Implantable or microcapsular sustained release matrices include polylactides (US Patent No:3,773,919, EP-A-0058481) copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al, Biopolymers 22(1): 547-556, 1985), poly(2-
15 hydroxyethyl-methacrylate) or ethylene vinyl acetate (Langer et al, J. Biomed. Mater. Res. 15:167-277, 1981, and Langer, Chem. Tech. 12:98-105, 1982). Liposomes containing the polypeptides are prepared by well-known methods: DE 3,218,121A; Epstein et al, PNAS USA,
20 82:3688-3692, 1985; Hwang et al, PNAS USA, 77:4030-4034, 1980; EP-A-0052522; E-A-0036676; EP-A-0088046; EP-A-0143949; EP-A-0142541; JP-A-83-11808; US Patent Nos 4,485,045 and 4,544,545. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type
25 in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal rate of the polypeptide leakage.

The 791Tgp72 and/or peptides of the CD55 family may be administered in a localised manner to a tumour site or
30 other desired site or may be delivered in a manner in which it targets tumour or other cells.

Targeting therapies may be used to deliver the active agent more specifically to certain types of cell,

by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons, for example if the agent is unacceptablely toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Instead of administering these agents directly, they may be produced in the target cells by expression from an encoding gene introduced into the cells, e.g. in a viral vector (a variant of the VDEPT technique - see below). The vector may be targeted to the specific cells to be treated, or it may contain regulatory elements which are switched on more or less selectively by the target cells.

The agent may be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT or VDEPT, the former involving targeting the activating agent to the cells by conjugation to a cell-specific antibody, while the latter involves producing the activating agent, e.g. an enzyme, in a vector by expression from encoding DNA in a viral vector (see for example, EP-A-415731 and WO 90/07936).

Vectors such as viral vectors have been used in the prior art to introduce nucleic acid into a wide variety of different target cells. Typically the vectors are exposed to the target cells so that transfection can take place in a sufficient proportion of the cells to provide a useful therapeutic or prophylactic effect from the expression of the desired polypeptide.

A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see US Patent No. 5,252,479 and WO 93/07282. In particular, a number of

viruses have been used as gene transfer vectors, including papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses. Many protocols in the prior art have used disabled murine
5 retroviruses.

As an alternative to the use of viral vectors other known methods of introducing nucleic acid into cells includes electroporation, calcium phosphate co-precipitation, mechanical techniques such as
10 microinjection, transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer.

Receptor-mediated gene transfer, in which the nucleic acid is linked to a protein ligand via polylysine, with the ligand being specific for a receptor
15 present on the surface of the target cells, is an example of a technique for specifically targeting nucleic acid to particular cells.

The vaccination dose of the 791Tgp72 or CD55 family polypeptide will be dependent upon the properties of the
20 vaccine employed, e.g. its binding activity and in vivo plasma half-life, the concentration of the polypeptide in the formulation, the administration route, the site and rate of dosage, the clinical tolerance of the patient involved, the pathological condition afflicting the
25 patient and the like, as is well within the skill of the physician. For example, doses of 300 μ g of polypeptide per patient per administration are preferred, although dosages may range from about 10 μ g to 1 mg per dose. Different dosages are utilized during a series of
30 sequential inoculations; the practitioner may administer an initial inoculation and then boost with relatively smaller doses of vaccine.

The vaccine compositions of the invention can be

administered in a variety of ways and to different classes of recipients. Examples of types of cancer that can be treated with the vaccine include colorectal cancer, osteosarcoma, breast and ovarian cancers.

5 This invention is also directed to optimized immunization schedules for enhancing a protective immune response against cancer. By way of example, at least three separate inoculations with 791Tgp72 and/or CD55 family polypeptides be administered, with a second
10 inoculation being administered more than two, preferably three to eight, and more preferably approximately four weeks following the first inoculation. It is preferred that a third inoculation be administered several months later than the second "boost" inoculation, preferably at
15 least more than five months following the first inoculation, more preferably six months to two years following the first inoculation, and even more preferably eight months to one year following the first inoculation. Periodic inoculations beyond the third are also desirable
20 to enhance the patient's "immune memory". See Anderson et al, J Infectious Diseases 160 (6):960-969, Dec.1989 and the references therein. Generally, infrequent immunizations with polypeptides spaced at relatively long intervals is more preferred than frequent immunizations
25 in eliciting maximum antibody responses, and in eliciting a protective effect.

 The above discussion, insofar as it relates to vaccine compositions and to the production of nucleic acid compositions, is generally applicable also to the
30 nucleic acid vaccines of the present invention, in accordance with the following comments which relate specifically to such vaccines.

 Nucleic acid immunisation involves the use of a

nucleic acid, usually DNA, vector encoding a gene of interest. A preferred vector is pcDNA3 (Invitrogen, Groningen, Netherlands). A DNA sequence encoding the gene of interest is typically placed under the control of a eukaryotic promoter that allows for expression in the target mammalian cells. By inclusion of various known sequence tags the encoded gene product may be directed to various compartments within the cell. This may be used to influence the direction of the developing immune response, for example favouring CTL or antibody responses.

The vector is introduced into the mammalian body by a number of possible routes. For example, injection of a naked DNA vector into muscle or via an intradermal route has been successful in establishing immune responses, a typical protocol involving the intramuscular injection of 50 μ g DNA into two muscles on three occasions. Other possible routes include encapsulation of the nucleic acid vector into particles that are taken up by antigen-presenting cells. Poly(lactide-coglycolide) PLG microparticles have been successfully used to raise immune responses by feeding the particles to mice.

A major advantage of nucleic acid immunisation is the prolonged production of immunogen from within the cells of the immunised mammal, in a similar way to that of viral infections. The vector nucleic acid has also been shown to be a stimulator of innate immunity, providing the right environment in which to establish an efficient and sustained immune response.

Example 1

Identification of 791Tgp72 Antigen by Immunoprecipitation

To improve the yield of 791Tgp72 antigen, both

immunoprecipitation and affinity chromatography
biotinylation of cell membranes was used to optimise the
purification protocol, enabling efficient tracing of
purified fractions. Cell culture and surface
5 biotinylation were carried out as described in Altin et
al, 1994. The cell line 791T was cultured in RPMI 1640
medium supplemented with heat-inactivate 10% fetal calf
serum (FCS). The cells were harvested with trypsin/EDTA
and washed three times with ice-cold PBS-C/M before
10 reacting with 0.5 mg/ml sulfo-NHS-biotin (Pierce) for 30
minutes at 4°C. In some experiments, biotinylation was
carried out in the presence of the chemical cross-linking
agent 3,3-dithio-bis(sulfo-succinimidyl-propionate)
(DTSSP; Pierce) to covalently link associated molecules.
15 For these studies, cells were suspended in phosphate
buffered saline minus $\text{CaCl}_2/\text{MgCl}_2$ (PBS-C/M, pH 7.6),
biotinylated and then crosslinked for 1 hr at room
temperature with gentle mixing, following the
manufacturers recommendations (PIERCE). Initial
20 precipitations were carried out on biotinylated samples.
Antibody (791T/36) was added to either whole cells or
cell lysates. For these experiments 1143/B7 Mab was used
as the negative control antibody.

Cells ($2-5 \times 10^7$) were lysed for 2 hrs at 4°C, cell
25 lysates were cleared by centrifugation at 13000 rpm for
15 minutes. Immune complexes were then formed with
protein-A sepharose (Sigma) for 30 minutes at 4°C. This
basic protocol allowed us to vary the detergents and
their concentrations, washing conditions and incubation
30 times in order to optimise the purification protocol.
Detergents tested were 0.5, 1.0 and 1.5 %; Nonidet P-40,
Tween-20 and Octyl Glucoside. These were used in TNE
(20mM Tris, pH 8.0, 140 mM NaCl, 5mM EDTA). Washes were

carried out with (20mM Tris pH 8.0, 100 mM NaCl, 1mM EDTA, 0.1 mM PMSF + 0.25 % detergent). Washed protein-A sepharose beads were boiled in sample buffer (+/-) mercaptoethanol, reducing or non-reducing conditions.

5 The samples were analysed by SDS-PAGE, Western blotted onto nitrocellulose membrane (HybondTM-C; Amersham), and detection of biotinylated proteins were carried out as described in Laemmli (1970), Stern (1993) and Dunbar (1994).

10 After transferring, the membrane was briefly washed with PBS and dried for 30 minutes at room temperature before blocking with PBS containing 0.1% Tween-20 and 1% BSA. The membrane was then washed twice for 5 minutes with PBS containing 0.1% Tween-20 and then incubated with

15 horseradish peroxidase (HRPO)-streptavidin (1:1500; GIBCOBRL) for 1 hour at room temperature. The membrane was then washed three times (as above) and proteins were detected using the enhanced chemiluminescence (ECL) protein detection system (Amersham) by exposing the

20 chemiluminescent blot to X-OGRAPH film. The detection of non-biotinylation proteins was carried out by silver staining.

Figure 1 shows the results of immunoprecipitation by mAb 791/36 from 791T cells. All cells were solublised in

25 TNEN buffer containing 1% NP-40. The binding reaction was carried out before (Lanes 1-4) or after (Lanes 5-8) cell solubilisation. Cross linking reagent (DTSSP) was used in some of the reactions (Lanes 3, 4, 7 and 8) and the precipitation was carried out using 79T/36 (odd lane

30 numbers) or 1143/B7 control antibody (even lane numbers). It can be seen that crosslinking the antibody to the cell surface (Lane 3) improved the amount of purified antigen compared to cells solublised without

crosslinking. Comparable results were obtained with cells solubilised prior to antibody addition (lanes 5 and 7).

5 **Example 2**

Checking the Conditions for Purification of the Antigen

 In order to optimise the conditions for the purification of large amounts of 791Tgp72 the conditions were varied as for purification of the biotinylated protein. Initially, CNBr-activated sepharose 4B was used to make an affinity column with 791T/36 Mab (see Hole et al, 1988; Hole et al, 1990; Goding, 1996), but this proved very inefficient. A modification of this procedure using Protein A sepharose was introduced (Scneider et al, 1982). 1-2 x 10⁹ 791T cells were solubilized in 100 ml of 1% octyl glucoside in Tris buffer (20 mM Tris-HCl pH 8.5, 150 mM NaCl, 25 mM benzamidine, 5 mM EGTA, 10 ug /ml leupeptin, 0.1 mM PMSF) for 1 hr at 4°C with continual mixing. Unsolubilized material was discarded after centrifugation at 13000 rpm for 10 minutes, this was followed by a 100,000 g centrifugation of the supernatant for 30 minutes. The solubilized material was loaded on to the protein-A Sepharose-791T/36 crosslinked affinity column with a flow rate of 0.3-0.4 ml/min. The column was then washed with 20 ml 50 mM Tris-HCl pH 8.0 containing 0.3 M NaCl with 0.1% NP-40. The 791Tgp72 antigen was eluted with 5 column volume of 50 mM diethylamine pH 11.5 containing 0.5% NP-40. The sample was immediately neutralised by adding 200 ul of 1 M Tris-HCl pH 8.0. The original sample was recycled over the column another 2-3 times as above to recapture any unpurified antigen. Fractions were assessed by SDS-PAGE and silver staining.

Figure 2 shows the effects of various conditions on purification efficiency. Lane 2-4 represent three cell lysates solubilized by different non-ionic detergents. Octyl-B-glucoside yielded more precipitates (lane 2) and ultracentrifugation when used achieved significant improvements in reducing background protein contamination. Using the 791T36-protein-A sepharose column and similar conditions we were able to show significant improvements in yields of antigen (Figure 3, lane 10). However, we also showed that antigen was also eluted by even the mildest washing conditions (Figure 3, lane 3-9)

Following analysis of the purification procedures, the final conditions were chosen for affinity chromatography:

- (1) Lysis buffer with 1% octyl-B-glucoside, pH 8.5 for 1 hour at 4°C.
- (2) Lysate centrifugation: 13000 rpm x 10 min following 100,000 g x 30 min.
- (3) Addition of cleared lysate to Protein A sepharose coupled to 791T/36 affinity column.
- (4) Cycle the supernatant over the column at 0.3-0.4 ml/min.
- (5) Washing the column with 20ml 20 mM Tris-HCl pH 8.0 containing 0.3 M NaCl and 0.1% NP-40.
- (6) Sample was eluted in 5 column volumes of diethylamine pH 11.5 containing 0.5% NP-40 and neutralised with 1M Tris.
- (7) The sample solution was recycled for 3-4 times to recover as much antigen as possible.

Example 3

Sequence Analysis

To determine the N-terminal amino acid sequence, affinity purified 791Tgp72/66 was concentrated using vivaspin centrifugation columns. Approximately 10 μ g of protein was analysed by SDS-PAGE and Western blotted onto
5 PVDF membrane (PROBLOT, ABI) following the manufacturer's recommendations, with a modification by addition of 0.1% SDS. Following transfer for 1-2 hrs, the blot was stained with Coomassie blue for 30 seconds and rinsed in 10 % methanol 20% acetic acid. The stained 66 and 72 KDa
10 bands were excised from the blot and subjected to 16 rounds of automated protein sequencing on an ABI XXX sequencer.

Figure 4 shows the results of silver staining from the fractions of protein A column. The antigens of
15 791Tgp72 and p66 were eluted in 2-3 fractions.

N-terminal sequence analysis gave the following sequence "DCGLPPDVPNAQPALE" which showed 100% identity with the sequence of decay accelerating factor (DAF, CD55).

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Example 4

Transfection of CD55 into CHO Cells

To check the recognition of CD55 by 791T/36 Mab, CHO cells were transfected with a CD55 cDNA clone. The clone
25 was obtained from Dr Dale Christiansen (Austin Research Institute, Victoria 3084, Australia). Cells transfected with the clone were assayed by FACS analysis for binding of anti-CD55 antibodies, 110 and BRIC 216 and also for binding of 791T/36. All the antibodies show good
30 binding to the CHO cells transfected with CD55 but no binding to untransfected cells, see Table 1.

Example 5

Flow Cytometry Anti-CD55 Binding Assay

To measure expression of 791Tgp72 and CD55 on tumour and primary cell lines, 2×10^5 of 791T, human umbilical vein endothelial cells (HUVEC) and erythrocytes were
5 mixed with cold anti-CD55 110, BRIC 216, 220, 791T/36 and control mAb 708 (0.1 μ g) separately at 37°C for 1 hr. Then rabbit anti-mouse FITC (1:100, DAKO, Denmark) was added to each tube and incubated for another 1 hr. Direct binding of 791T/36 FITC (0.1 μ g) to 791T cells was
10 measured after 1hr at 37°C. The cells were washed two times with RPMI 1640 medium, fixed and measured by flow cytometry.

Table 2 present the results of antibodies binding to different cell lines. The data show that both anti-CD55
15 and 791T/36 mabs bind to red blood cells, HUVEC cells and to the osteosarcoma cell line 791T. The anti-CD55 antibody BRIC 216 bound most strongly to red blood cells and HUVEC cells whereas 791T/36 showed the strongest binding to 791T cells which was approximately 2 orders of
20 magnitude higher than to the normal cells. These results suggest that 791Tgp72 is closely related to CD55 but that there are some differences. These differences could be differential glycosylation or post-translational modifications, e.g. point mutations.

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Example 6**Immunoprecipitation with Various Anti-CD55 Antibodies**

To confirm whether anti-CD55 monoclonal antibodies could precipitate an antigen from tumour cells, the same
30 immunoprecipitation protocol as mentioned previously was used. 40 μ g of anti-CD55 110, BRIC 216 and anti-791Tgp72, 91T/36 were used to precipitate the antigen from 2×10^7 791T cells respectively.

Both anti-CD55 monoclonal antibodies, 110, BRIC 216 and 791T36 immunoprecipitated an antigen of similar molecular weight although the yield was far greater with 791T/36 than the with the anti-CD55 antibodies (Figure 5). These results again suggest that a similar antigen is precipitated by both the anti-CD55 antibodies and 791T/36, but that the later Mab has either better access or a higher affinity for 791Tgp72.

10 Example 7

Phosphatidylinositol Phospholipase C (PI-PLC) Treatment

CD55 is a GPI linked protein. To confirm whether 791Tgp72 is also GPI linked, 791T cells were treated with Phosphatidylinositol phospholipase C (PI-PLC; Boehringer Mannheim, Germany), to release GPI linked antigens. Cells (5×10^5) were incubated with PI-PLC(1 U/ml) for 1 hour at 37°C. The cells were washed two times with PBS and the expression of CD55 and or 791Tgp72 was determined by indirect immunofluorescence binding with monoclonal antibodies and flow cytometric analysis.

As shown in Table 3, the binding of anti-CD55 monoclonal antibodies and 791T/36 decreased after incubation with PI-PLC for 1 hr, with a maximal decrease in surface expression of approximately 85-90%. Cells incubated in parallel without PI-PLC retained their surface expression of 791Tgp72 antigen. These results clearly show that 791Tgp72 is also GPI linked.

Example 8

30 Purified 791Tgp72 Antigen

To confirm that the anti-CD55 mabs can bind to 791Tgp72, purified antigen (50 ng) or antigen released from PI-PLC treated 791T cells was added separately to

flexible microtest plates (Falcon, Becton Dickinson, CA, USA) and left at 37°C till dry. The plates were washed three times with phosphate buffered saline containing 0.05% Tween-20 (PBS-Tween) and blocked with BSA (1%) for 1 hr at room temperature. The plates were washed three times, then anti-CD55 antibodies (500 ng) in PBS were added. After 1hr at room temperature the plates were washed three times in PBS-Tween and conjugated rabbit anti-mouse horseradish peroxidase (HRPO) diluted 1:1000 was added for a further 1hr. Finally, after extensive washing, the plates were developed and read at 405 nm.

The binding of 791Tgp72 antigen to mAb 791T/36 and other anti-CD55 antibodies was shown by ELISA. The binding of both 791T/36 and anti-CD55 antibodies to purified 791Tgp72 antigen was clearly seen. 791T/36 also showed significant binding to 791Tgp72 antigen released from PI-PLC treated 791T cells (Figure 6). The binding of the anti-CD55 mAbs to purified 791Tgp72 confirm that this antigen shares considerable homology with CD55.

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Example 9

Mapping of the 791T/36 Epitope

DAF (CD55) consists of 4 SUSHI domains, a C-terminal O-glycosylated tail and a GPI anchor. Purified 791Tgp72 was used in a sandwich ELISA to determine to which domain 791T/36 bound. The antigen was captured with either one of the anti-CD55 Mabs or 791T/36 and then detected with 791T/36. Thus, recognition of the antigen by the same antibody as the capture antibody would indicate that the antibody is able to bind to two sites on the purified 791Tgp72. Conversely, absence of binding would indicate that the antibody has only one binding site on the antigen. In this way, antibodies can be mapped to the

different domains of CD55. Plates were coated with anti-CD55 antibodies 220 (SUSHI domain 1), 110 (SUSHI domain 2), BRIC 216 (SUSHI domain 3) and left at 4°C overnight. The plates were washed three times with PBS-Tween and
5 blocked with BSA (1%) for 1 hr at room temperature. The plates were washed three times, then purified 791Tgp72 antigen (25ng) was added. After 1 hr at room temperature, the plates were washed three times and biotinylated mAb 791T/36 (500ng per well) was added.
10 Following incubation at room temperature for 1 hr, and washing three times, streptavidin-HRPO diluted 1:1000 was added for a further 1 hr. After a further six washes, the plates were developed and read at 405 nm.

Figure 7 shows that the 791Tgp72 antigen captured by
15 monoclonal antibodies which bound to SUSHI domains 1 and 3 could be detected by 791T/36 biotin. Interestingly, capture of 791Tgp72 by mAb 110, which was raised against SUSHI domain 2, or 791T/36 could not be detected by 791T/36 biotin, suggesting that 791T/36 must bind near
20 SUSHI domain 2.

The anti-CD55 antibodies were tested in a competition assay for their ability to inhibit the binding of 791T/36 to 791T cells. The inhibition of 791T/36 binding would indicate that the competing
25 antibody bound to a similar or shared antigenic site on the 791Tgp72 molecule.

791T cells (2×10^5) were mixed with different amounts of cold anti-CD55 monoclonal antibodies at 37°C for 30 minutes prior to adding mAb 791T/36 FITC (0.1 µg).
30 After 1 hr at 37°C, the cells were washed two times with RPMI 1640 medium, fixed and measured by flow cytometry.

Table 4 shows that in the competition binding assay

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of anti-CD55 with 791T/36 FITC, only cold mAb 791T/36 inhibited the binding of labelled 791T/36. These results suggest that although 791T/36 binds at or near SUSHI domain 2, it binds at a distinct site to monoclonal antibody 110.

Example 10

CHO Transfections with CD55/CD46 Chimeric Proteins

In order to ascertain the domain to which 791T/36 binds, a number of chimeric constructs were made comprising CD46, a membrane bound complement control protein with similar structure to CD55, i.e. contains four SUSHI domains but those domains are distinct to those of CD55. The constructs were produced by Dr Dale Christiansen (Austen Research Centre, Victoria, Australia). The constructs tested were:

- (1) CD46 (CD55 3); CD46 with SUSHI domain 3 substituted with that of CD55.
- (2) CD46 (CD55 4); CD46 with SUSHI domain 4 substituted with that of CD55.
- (3) CD46 (CD55 3/4); CD46 with SUSHI domains 3/4 substituted with those of CD55.
- (4) CD46 (CD55 1/2); CD46 with SUSHI domains 1/2 substituted with those of CD55.

Only CHO cells transfected with constructs containing CD55 SUSHI2 showed significant binding to 791T/36 monoclonal antibody (Table 1).

Example 11

Anti-Idiotypic Antibodies

A human (105AD7) and a mouse (730) anti-idiotypic antibodies which bind at the antigen combining site of 791T/36 have been produced. A competition assay was used

to assess if these anti-idiotypic antibodies could also bind to the other anti-CD55 antibodies. 791T cells (2×10^5) were mixed with anti-CD55 (0.1 μ g) and varying amounts of 105AD7 or 730 at 37°C for 1hr. The cells were
5 washed two times with RPMI 1640 medium prior to the addition of rabbit anti-mouse FITC (1:100) for a further 1hr. The cells were washed two times with RPMI 1640 medium, fixed and measured by flow cytofluorometry.

The results in tables 5 and 6 indicate the binding
10 of 791T/36 to 791T cells decreased when increasing concentration of mAb 105AD7 or 730 were added. In contrast, no loss in binding of the other anti-CD55 was seen in the presence of either anti-idiotypic. These results add support to the conclusion that 791T/36 is a
15 unique anti-CD55 monoclonal antibody.

The anti-idiotypic antibodies both stimulate humoral and cellular responses against cells which express 791Tgp72 antigen suggesting that they can mimic the antigen. Comparison of the amino acid sequences of both
20 anti-idiotypes with CD55 show areas of homology with both CDRH3 regions of the antibodies and distinct regions of SUSHI domain 2.

For 105AD7:

CDR L1 - homology 7/9 amino acids with SUSHI1 83-93.

25 CDH H3 - homology 5/7 amino acids with SUSHI2 151-158.

For 730:

CDR L1 - homology 6/9 amino acids with SUSHI1 83-93.

CDH H3 - homology 5/7 amino acids with SUSHI2 121-128.

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Example 12

Western Blotting

To confirm that 791T/36 could immunoprecipitate CD55

from normal cells, erythrocytes (2×10^9) were washed two times with PBS and solubilized by NP-40 (1%, 10 ml). After centrifugation at 3000 rpm for 10 minutes, the supernatant was removed to a clean tube for

5 centrifugation ($100,000g \times 30 \text{ min}$). $10 \mu\text{l}$ of erythrocyte supernatant (equal to 2×10^6 of erythrocytes) and purified 791Tgp72 antigen (200 ng) was loaded onto SDS-PAGE at non-reducing condition as described previously. Proteins were transferred to nitrocellulose membrane and blocked

10 with PBS containing BSA (1%) for 1 hr at room temperature (RT). After two times wash with PBS-Tween (0.1%), primary antibody was added for 1 hr at RT. The blots were washed two times and rabbit anti-mouse conjugate diluted 1:1000 was added. Following 1 hr incubation and

15 extensive washing, the blots were developed by ECL system.

Detecting the 791Tgp72 antigen from erythrocytes and 791T cells by Western blotting indicated some differences. Only one band at 72 kDa was found on

20 erythrocytes whereas two bands of 72 and 66 kDa exist on 791T cells (Figure 8).

Treatment of the 72 kDa and 66 kDa bands with neuraminidase (which removes sialic acid residues from glycoproteins) yielded in each case a band of 55 kDa,

25 suggesting that the 72 kDa and 66 kDa proteins are glycosylation variants of each other.

Example 13

Clone and DNA Sequence

30 To confirm the identity of 791Tgp72, the gene encoding this protein was cloned and sequenced. Total cellular RNA was isolated by the guanidine isothiocyanate method from 791T cells (4×10^7) grown in monolayers.

First-strand cDNA synthesis was carried out using Ready-To-Go First-Strand Kit (Pharmacia Biotech, UK). Primers based on the N-terminal protein sequence obtained from the 72 and 66 KDa bands were generated;

5 Pep 5': GACTGTGGCCTTCCCCCAG
 C-CD55-5': AAAATGACCGTCGCGCGGCCG
 C-CD55-3': CTAAGTCAGCAAGCCCATGGT
 B-CD55-5': GAATACTGCAGATGACCGTCGCGCGGCCG
 B-CD55-3': CCTACGAATTCTAAGTCAGCAAGCCCATGG
 10 FL-CD55-3': ATGTGATTCCAGGACTGCC
 FL-CD55-5': TGGGCGTAGCTGCGACTCG

These primers were designed for the following:

15 C-CD55: Cloning and expression in eukaryotic cells
 from the recognised start codon to the stop codon of
 native CD55.

20 B-CD55: Cloning and expression into a bacterial
 expression vector in order to generate protein for
 purification. The sequences include the addition of a 5'
 EcoRI site and a 3' PstI site.

 FL-CD55: were designed for cloning of the recognised
 coding region of CD55 and 200 bp of the 3' untranslated
 region. This should allow the cloning of potential
 splice variants that occur in the 3' end of the antigen.

25 791Tgp72 PCRs were set up with first strand cDNA ,
 the primers used were mixes of the primer sets outlined
 above. The samples were placed in a thermal cycler, the
 following profile was used (hot start at 94°C for 2
 minutes; denaturation at 94°C for 30 seconds, 55°C for 45
 30 seconds, 72°C for 90 seconds, repeat for a total 30
 cycles). PCR products were cloned into modified
 pBluescript SK-vector. Positive clones were checked by
 PCR using vector specific primers and the positive DNA

plasmids sequenced on an ABI automated sequencer.

The initial cloning experiments resulted in products generated from PEP5' and either CCD553', BCD553' or FLCD553'. The results of this sequencing revealed there
5 to be no difference in sequence between the cloned products and the full reported sequences of CD55 (figure 9). The translated amino acid sequence of CD55 is set out in figure 10.

Recently full length versions of the PCR generated
10 CD55 products have been cloned using FLCD555' and either FLCD553', CCD553' or BCD553'.

Discussion

Interest in the use of 791Tgp72 as a target for
15 immunotherapy arose initially by the demonstration that this antigen was expressed by the majority of osteosarcomas, colorectal, gastric and ovarian tumours. The tumour specificity of 791Tgp72 was also emphasised by extensive clinical imaging studies with radiolabelled
20 anti-791Tgp72 monoclonal antibody 791T/36, in the detection of primary and metastatic colorectal cancer, osteosarcoma, breast and ovarian cancer. The results shown herein relate to the first isolation of 791Tgp72 and the use of this antigen or a related family member
25 CD55 as a cancer vaccine.

From the prior art, CD55 is a very surprising target for T-cell immunity as it is expressed on essentially all haematopoietic cells and on endothelial and epithelial
30 tissues, including the vascular endothelium, gastrointestinal tract, genitourinary tract, central nervous system, and extracellular matrix. 791T/36 binds weakly to erythrocytes and it may be that this has been advantageous in the clinical imaging studies. 791T/36

antibody may have bound weakly to erythrocytes which upon passage through the tumour have allowed transfer of the antibody to 791Tgp72 to which it binds with higher affinity. CD55 was initially purified based on its ability to accelerate the decay of the classical pathway C3 convertase, C4b2a. It carries out the same function with respect to the alternative pathway C3 convertase, C3bBb, but does not have any cofactor activity for the factor I-mediated proteolytic degradation of C3b or C4b. So CD55 protects the cell from complement-mediated lysis at the C3 convertase step.

Normal human tissues express membrane-associated complement inhibitory proteins that protect these tissues from damage by autologous complement. To determine whether neoplasms also express these proteins prior investigators have examined the distribution of CD55 (DAF), CD59 (protectin) and CD46 (membrane cofactor protein) in frozen samples of human breast, colon, kidney, and lung carcinomas and in adjacent non-neoplastic tissues. Difference between normal tissues and the corresponding neoplasms were observed, with loss or gain of expression of one or more inhibitors. Some tumours expressed only one inhibitor whether others expressed different combinations of two or three inhibitors. Colon carcinomas, by contrast, expressed all inhibitors. The results demonstrate that most carcinomas, with the exception of small cell carcinomas of the lung, do express one or more complement inhibitors at a level likely to inhibit complement-mediated cellular damage. Other tumour tissues, such as ovarian and gastrointestinal tumour cells, were also checked. The surface expression level of CD55 varied, and correlation with the vulnerability of the cells to C-mediated lysis.

Thus, the expression of C regulators on malignant cells may constitute a tumour escape mechanism, and is a critical parameter to be examined when mAb therapy is being considered. Furthermore, expression of CD55 on target cells makes them resistant to lysis by natural killer cells. Many tumours escape T-cell recognition by loss of MHC molecules, however this makes them susceptible to NK killing. Over-expression of CD55 which inhibits NK lysis is therefore an obvious advantage.

The extensive expression of CD55 on normal cells, its role in protecting cells from complement and NK lysis makes a very unlikely target for T-cell immunotherapy. However, clinical trials with 105AD7 which mimics an epitope on CD55 are showing that it can stimulate excellent T-cell responses. 791Tgp72 does however show some differences from CD55. Two bands are precipitated from tumour cells whereas only one band is seen in erythrocytes. Although the anti-CD55 antibodies can precipitate the 791Tgp72 from tumour cells the yield is much lower than is observed with 791T/36. This is reflected in the cell binding assays where 791T/36 shows strongest binding to tumour cells whereas the anti-CD55 monoclonal antibody BRIC 216 binds better to erythrocytes. Different forms of CD55 have been isolated from tissue such as erythrocytes, urine and tears (Nakano et al, 1991; Sugita et al, 1988; Seya et al, 1995). CD55-A (63kDa) and CD55-B (55kDa) from erythrocytes do not appear to have a GPI anchor. CD55-U2 (60-80kDa) in urine is thought to be inactive. The existence of a human splice variant of CD55 has been suggested but the putative protein has never been isolated. Furthermore, new functions other than complement decay have been suggested. Activated T-cells

which have been crosslinked with anti-CD55 monoclonal antibodies can induce T-cell proliferation and signal transduction. It is unclear if this is related to the recent observation that CD55 is the ligand for the CD97
5 receptor expressed on activated T-cells.

Whether there are different roles for CD55 or different forms of CD55/791Tgp72 in tumour cells or whether there is differential it remains an interesting prospect to use a molecule which tumours over-express to
10 protect themselves from immune attack as a cancer vaccine. The dichotomy being that if the cell fails to express the molecule it is susceptible to complement mediated and NK lysis and if it does express the antigen it will be killed by CD55 specific T-cells.

References

The references mentioned herein are all expressly incorporated by reference.

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Table 1.

Fluorescence of Mabs on chimeric transfectants			
Constructs	791T/36	1H4	E4.3
wt DAF	105	164	16
DAF 3	4	94	79
DAF 4	3	-	70
DAF 3/4	5	-	70
DAF 1/2	108	-	3

(a) 1H4 Mab binds to sushi domain 3 of DAF

(b) E4.3 Mab binds to sushi domain 1 of CD 46

Chimeric constructs were transfected into CHO cells and assayed for their binding of 791T/36, 1H4 and E4.3.

Table 2.**Indirect Immunofluorescence Assay Of Various Antibodies
On DAF Positive And Negative Cells**

Mean Linear Fluorescence			
Antibody	Erythrocytes	791T	HUVEC
110	52.71	1186.96	109.33
216	94.28	1776.41	172.52
791T/36	60.07	2373.89	146.96
Rabbit anti- mouse FITC	26.68	27.88	55.55
708	24.11	36.42	47.38

Monoclonal antibodies to DAF (110, 216) and 791Tgp72 (791T36) were used to label a range of cells for 1 hour at 4°C. Cells were then incubated with FITC-labelled rabbit anti mouse antibody and read by FACS. Analysis was also carried out using Mab 708 as a negative control and with Rabbit anti-mouse FITC alone.

Table 3.

Binding Of 791T/36, Control IgG2b (708) And Anti-DAF Antibodies To 791T Cells Following Treatment With PI-PLC At 1u/ml For 1 Hour.

Antibodies	PI-PLC (-)	PI-PLC(+)	% Inhibition
110	963.40	86.34	91
216	1101.51	208.83	82
791T/36	1991.35	178.6	91
708	29.79	25.1	14

Mean linear fluorescence readings of anti DAF (110, 216) Mabs and anti 791T/gp72 (791T/36) antibodies to phospholipase treated or untreated 791T cells. 708 Mab was used as a control antibody.

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Table 4.

**Competitive Binding Of 791T/36 FITC (0.1ug) To 791T Cells By Anti DAF Antibodies,
Unlabelled 791T/36 Or An Irrelevant IgG2b Mab Which Does Not Bind To 791T Cells**

Cold Mab (ug)	708	220	110	216	791T/36	% Inhibition with 791T/36
0	453.82	450.96	459.65	502.11	473.21	0
0.1	426.73	505.04	488.02	456.44	335.41	30
0.5	451.60	499.91	493.05	496.46	162.38	66
1.0	454.20	455.08	497.82	509.80	103.47	79

FITC labelled 791T/36 antibody was incubated with various concentrations of the above unlabelled antibodies. Only unlabelled 791T/36 was able to inhibit binding of the FITC labelled 791T/36. Mean linear fluorescence readings are given.

Table 5.

Competition Assay Of 105AD7 With Various Anti-DAF Antibodies On 791T Cell

105AD7 (ug)	708	110	216	791T/36
0	15.03	489.87	803.19	912.46
0.05	14.84	528.65	701.71	1089.17
0.1	17.24	388.11	783.55	912.35
0.5	14.72	533.11	607.61	726.25
1.0	16.14	512.53	626.31	570.13
5.0	20.85	370.53	562.24	53.07

Anti-DAF antibodies (110, 216) and anti 791T/gp72 (791T/36) were added to 791T cells in the presence of increasing concentrations of 105AD7 anti-idiotypic antibody, which specifically recognises the binding site of 791T/36. Cells were incubated for 1 hour at 37°C then for a further hour in the presence of FITC-labelled Rabbit anti-mouse. Cells were analysed by FACS. 708 Mab was used as a negative control. The results indicate that only 791T/36 was inhibited by 105AD7. Mean linear fluorescence readings are given.

Table 6.

Competition Assay Of 730 With Various Anti-DAF Antibodies On 791T Cell

730 (ug)	708	110	216	791T/36
0	19.97	607	1356	1382
0.1	17.63	642	1185	1352
0.5	19.02	632	1158	983
1.0	19.31	620	1229	597
2.5	23.10	739	1212	89
5.0	32.54	640	1179	70

Anti-DAF antibodies (110, 216) and anti 791T/gp72 (791T/36) were added to 791T cells in the presence of increasing concentrations of Mab 730 anti-idiotypic antibody, which specifically recognises the binding site of 791T/36. Cells were incubated for 1 hour at 37°C then for a further hour in the presence of FITC-labelled Rabbit anti-mouse. Cells were analysed by FACS. 708 Mab was used as a negative control. The results indicate that only 791T/36 was inhibited by 730. Mean linear fluorescence readings are given.

CLAIMS:

1. A cancer vaccine comprising a polypeptide of the CD55 family, or a fragment or derivative of a polypeptide of the CD55 family, wherein the vaccine is capable of inducing an immune response in a patient.
2. A cancer vaccine according to claim 1 wherein the polypeptide of the CD55 family is, or the fragment or derivative is of, 791Tgp72 antigen.
3. A cancer vaccine according to claim 1 or claim 2 comprising full length 791Tgp72 antigen or CD55 polypeptide.
4. A cancer vaccine according to any preceding claim wherein the antigen, polypeptide, fragment or derivative has part or all of the amino acid sequence of Fig. 10.
5. A cancer vaccine according to any preceding claim wherein the antigen, polypeptide, fragment or derivative includes part or all of the amino acid sequence consisting of amino acids 97-159 of Fig. 10.
6. A cancer vaccine according to claim 5 wherein the antigen, polypeptide, fragment or derivative includes a sequence having at least five amino acids identical with corresponding amino acids of a contiguous stretch of seven amino acids contained within amino acids 121-128 or 151-158 of Fig. 10.
7. A cancer vaccine according to any preceding claim wherein the antigen, polypeptide, fragment or derivative

includes a sequence having at least six amino acids identical with corresponding amino acids of a contiguous stretch of nine amino acids contained within amino acids 83-93 of Fig. 10.

5

8. A cancer vaccine according to any preceding claim comprising a fragment of at least five contiguous amino acids from a polypeptide of the CD55 family.

10 9. A cancer vaccine according to claim 8 comprising a fragment of at least five contiguous amino acids from the amino acid sequence shown in Fig. 10.

15 10. A cancer vaccine according to claim 8 or claim 9 wherein the fragment is of at least seven contiguous amino acids.

11. A cancer vaccine according to claim 10 wherein the fragment is of at least nine contiguous amino acids.

20

12. A cancer vaccine according to claim 11 wherein the fragment is of at least 13 contiguous amino acids.

25 13. A cancer vaccine comprising a nucleic acid molecule which encodes an antigen, polypeptide, fragment or derivative as specified in any preceding claim, wherein the vaccine is capable of inducing an immune response in a patient.

30 14. A cancer vaccine according to claim 13 having part or all of a nucleic acid sequence as shown in Fig. 10 or Fig. 11.

15. A cancer vaccine according to any preceding claim wherein the immune response is one or more of a T-helper cell response, a cytotoxic T-cell response and a NK cell response.
- 5
16. A cancer vaccine according to any preceding claim which is capable of inducing an immune response against CD55 or 791gp72 as expressed by cancer cells.
- 10
17. A cancer vaccine according to claim 16 wherein the immune response has greater affinity for cancer cells than for non-cancerous cells.
- 15
18. The use of an antigen, polypeptide, fragment, derivative or a nucleic acid molecule as specified in any preceding claim in the preparation of a medicament for the treatment of cancer.
- 20
19. A method of treating a patient having cancer, the method comprising administering to the patient a therapeutically effective amount of a cancer vaccine as defined in any one of claims 1 to 17.
- 25
20. Isolated and purified 791Tgp72 antigen.
21. Isolated and purified 791Tgp72 antigen according to claim 20 having the amino acid sequence shown in Fig. 10 and a molecular weight of approximately 66 kD.
- 30
22. Isolated and purified 791Tgp72 antigen according to claim 20 or claim 21 wherein the specificity of antibody binding to the said antigen, relative to antibody binding to the CD55 antigen as expressed on human red blood cells

and/or HUVEC cells, is greater for 791T/36 than for anti-CD55 antibody BRIC 216.

23. Isolated and purified 791Tgp72 antigen according to
5 any one of claims 20 to 22, as obtainable by:

(a) solubilising 791T cells in lysis buffer
including 1% octyl-B-glucoside, pH 8.5 for 1 hour at 4°C;

(b) centrifuging the lysate at 13000 rpm x 10 min
following 100,000 g x 30 min;

10 (c) adding the cleared lysate to Protein A
sepharose coupled to 791T/36 affinity column;

(d) cycling the supernatant over the column at 0.3-
0.4 ml/min;

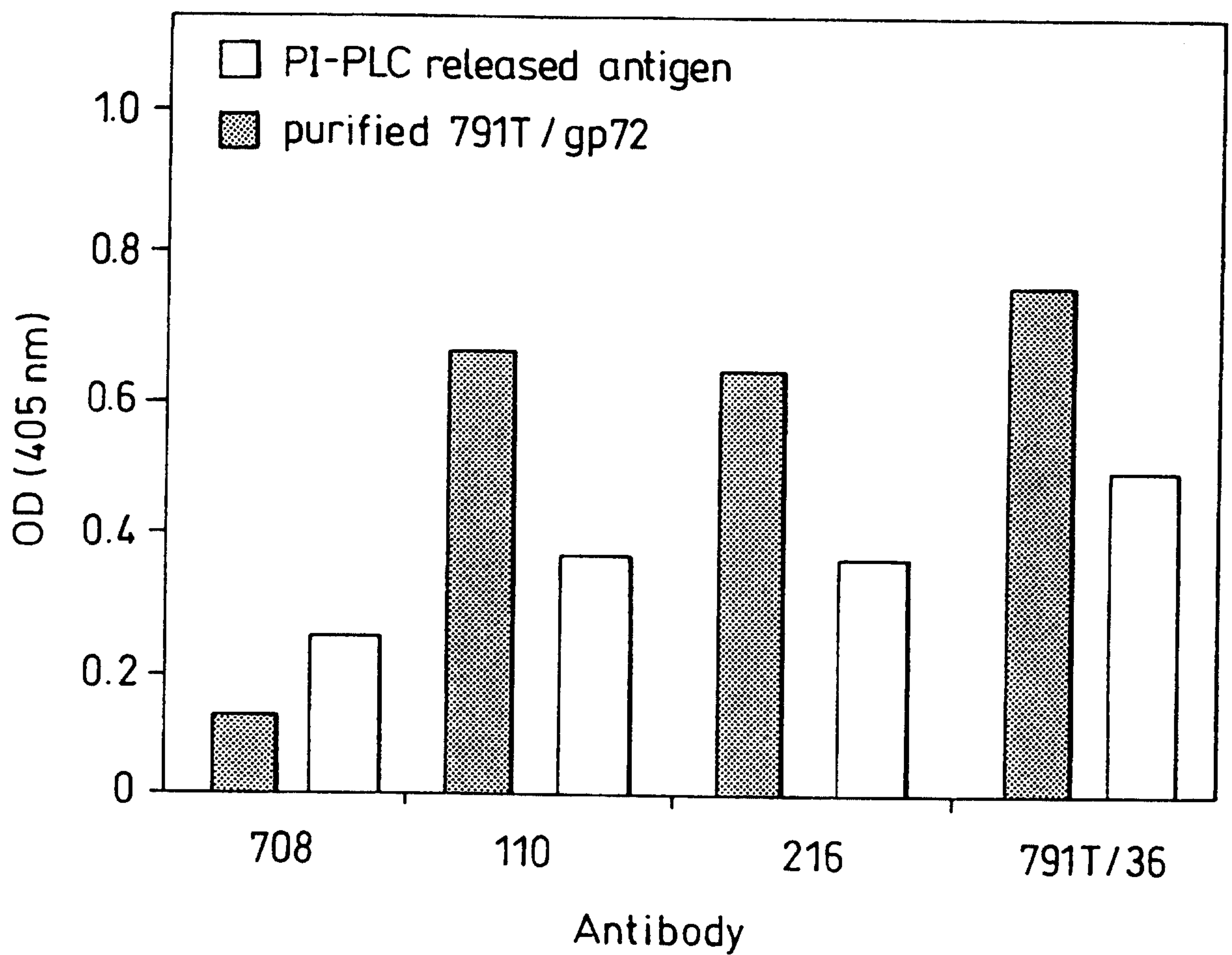
(e) washing the column with 20ml 20 mM Tris-HCl pH
15 8.0 containing 0.3 M NaCl and 0.1% NP-40; and,

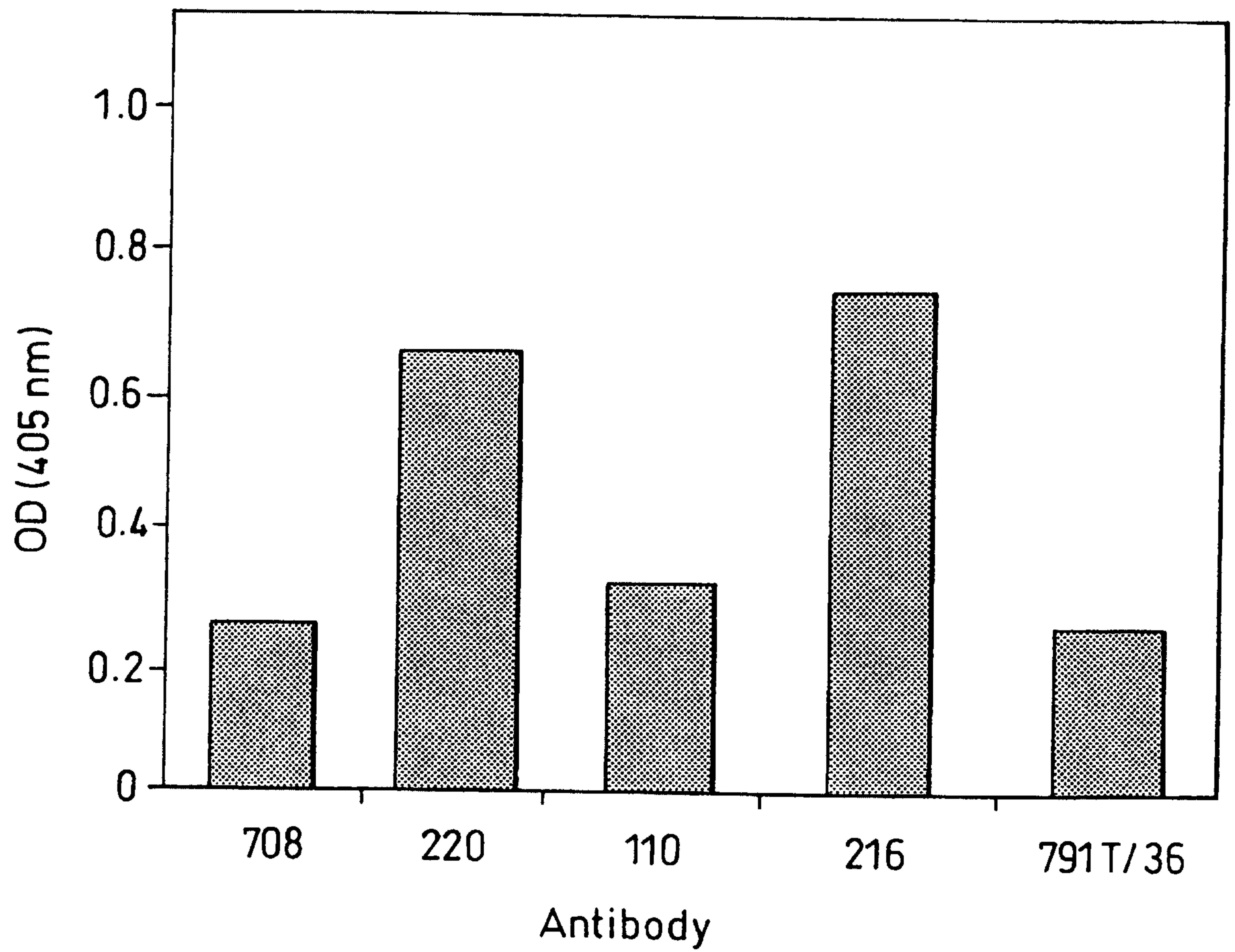
(f) eluting 791Tgp72 from the column in 5 column
volumes of diethylamine pH 11.5 containing 0.5% NP-40 and
neutralising the eluate with 1M Tris.

20 24. A pharmaceutical composition comprising 791Tgp72
according to any one of claims 20 to 23 in combination
with a pharmaceutically acceptable carrier.

25 25. 791Tgp72 according to any one of claims 20 to 23 for
use in a method of medical treatment.

26. A method for isolating 791Tgp72 antigen from cells
expressing 791Tgp72, the method including the steps of:
solubilising the cells with lysis buffer including
30 octyl-glucoside; and
treating the lysate using ultracentrifugation.

6/23***Fig. 6***

7/23***Fig. 7***

9/23

[illegible]

Fig. 9 (part 1 of 11)

10/23

180		190		200		210		220		230	
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5 / P5
C-DAF.seq
RC of B/C DAF

```

-----GTACAAGTTTCC
GCCTTCCCCAGATGTACCTAATGCCAGCCAGCTTTGGAAGGCCGTACAAAGTTTCC
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Year	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030
Population (millions)	10.0	10.1	10.2	10.3	10.4	10.5	10.6	10.7	10.8	10.9	11.0	11.1
GDP (trillion USD)	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0	2.1
Life expectancy (years)	75	76	77	78	79	80	81	82	83	84	85	86
Urban population (%)	55	58	61	64	67	70	73	76	79	82	85	88
Renewable energy (%)	10	12	15	18	22	26	30	34	38	42	46	50
Carbon emissions (Gt CO2e)	1.5	1.6	1.7	1.8	1.9	2.0	2.1	2.2	2.3	2.4	2.5	2.6
Healthcare spending (GDP %)	5.0	5.2	5.5	5.8	6.1	6.4	6.7	7.0	7.3	7.6	7.9	8.2
Education spending (GDP %)	3.0	3.1	3.2	3.3	3.4	3.5	3.6	3.7	3.8	3.9	4.0	4.1
Research & Development (GDP %)	2.0	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	3.0	3.1
Unemployment (%)	6.0	6.2	6.5	6.8	7.1	7.4	7.7	8.0	8.3	8.6	8.9	9.2
Income inequality (Gini Index)	35	36	37	38	39	40	41	42	43	44	45	46
Digital literacy (%)	40	45	50	55	60	65	70	75	80	85	90	95
Government debt (GDP %)	60	62	65	68	71	74	77	80	83	86	89	92
Immigration (net, million)	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6
Trade openness (%)	25	26	27	28	29	30	31	32	33	34	35	36
Environmental index	60	62	64	66	68	70	72	74	76	78	80	82
Gender equality index	70	71	72	73	74	75	76	77	78	79	80	81
Human Development Index	0.70	0.72	0.74	0.76	0.78	0.80	0.82	0.84	0.86	0.88	0.90	0.92
Corruption index	40	41	42	43	44	45	46	47	48	49	50	51
Trust in government (%)	50	51	52	53	54	55	56	57	58	59	60	61
Public safety index	65	66	67	68	69	70	71	72	73	74	75	76
Infrastructure index	70	71	72	73	74	75	76	77	78	79	80	81
Quality of life index	75	76	77	78	79	80	81	82	83	84	85	86
Environmental quality index	60	61	62	63	64	65	66	67	68	69	70	71
Social stability index	70	71	72	73	74	75	76	77	78	79	80	81
Economic growth (GDP %)	5.0	5.1	5.2	5.3	5.4	5.5	5.6	5.7	5.8	5.9	6.0	6.1
Unemployment (avg, %)	6.0	6.2	6.5	6.8	7.1	7.4	7.7	8.0	8.3	8.6	8.9	9.2
Income inequality (avg, Gini)	35	36	37	38	39	40	41	42	43	44	45	46
Digital literacy (avg, %)	40	45	50	55	60	65	70	75	80	85	90	95
Government debt (avg, GDP %)	60	62	65	68	71	74	77	80	83	86	89	92
Trade openness (avg, %)	25	26	27	28	29	30	31	32	33	34	35	36
Environmental index (avg)	60	62	64	66	68	70	72	74	76	78	80	82
Gender equality index (avg)	70	71	72	73	74	75	76	77	78	79	80	81
Human Development Index (avg)	0.70	0.72	0.									

5 / P5
C-DAF. seq
RC of B/C DAF

	300	310	320	330	340

5/P5
C-DAF. seq
RC of B/C DAF

AAGGACTCAGTGATCTGCCCTTAAGGGCAGTCAATGGTCAGATATTGAAGAGTTCTGCA
AAGGACTCAGTGATCTGCCCTTAAGGGCAGTCAATGGTCAGATATTGAAGAGTTCTGCA

Fig. 9 (part 2 of 11)

5/P5	350	360	370	380	390	400
C-DAF.seq						
RC of B/C DAF						
	ATCGTAGCTGCGAGGTGCCAACAAAGGCTAAATTCTGCATCCCTCAAACAGCCTTATAT					
	ATCGTAGCTGCGAGGTGCCAACAAAGGCTAAATTCTGCATCCCTCAAACAGCCTTATAT					

	410	420	430	440	450	460
	CACTCAGAAATTATTTCCAGTCGGTACTGTTGTGGAATATGAGTGCCGTCAGGTTAC					
	CACTCAGAAATTATTTCCAGTCGGTACTGTTGTGGAATATGAGTGCCGTCAGGTTAC					

	470	480	490	500	510	520
	AGAAGAGAACCTTCTCTATCACCAAACCTAACTTGCCTTCAGAAATTAAATGGTCCA					
	AGAAGAGAACCTTCTCTATCACCAAACCTAACTTGCCTTCAGAAATTAAATGGTCCA					

	530	540	550	560	570	580
	CAGCAGTCGAAATTTGTAAAAAGAAATCATGCCCTAATCCGGGAGAAATACGAAATGC					
	CAGCAGTCGAAATTTGTAAAAAGAAATCATGCCCTAATCCGGGAGAAATACGAAATGC					

Fig. 9 (part 3 of 11)

[illegible]

Fig. 9 (part 4 of 11)

5/P5	760	770	780	790	800	810
C-DAF.seq						
RC of B/C DAF						
	CAAATTGACA-TGGAAATAATCCAGGGGAACGTTGACCATTATGGATATAACG-----					
	CAAATTGACAATGGAAATAATTCAAGGGGAACGTGACCATTATGGATATAGACAGTCTG					
	CAAATTGACAATGGAAATAATTCAAGGGGAACGTGACCATTATGGATATAGACAGTCTG					
	820	830	840	850	860	870

5/P5	TAACGTATGCATGTAATAAAGGATTCACCATGATTGGAGAGCACTCTATTATTGTAC					
C-DAF.seq	TAACGTATGCATGTAATAAAGGATTCACCATGATTGGAGAGCACTCTATTATTGTAC					
RC of B/C DAF						
	880	890	900	910	920	

5/P5	TGTGAATAATGATGAAGGAGAGTGGAGTGGCCACCACCTGAATGCAGAGGAAATCT					
C-DAF.seq	TGTGAATAATGATGAAGGAGAGTGGAGTGGCCACCACCTGAATGCAGAGGAAATCT					
RC of B/C DAF						

Fig. 9 (part 5 of 11)

[illegible]

Fig. 9 (part 6 of 11)

5/P5	1170	1180	1190	1200	1210
C-DAF.seq					
RC of B/C DAF					

	TCACGTTGACAGGTTTGCTTGGGACGCTAGTAACCATGGGCTTGCTGACTTAGCCAAA				
	TCAC				

	1220	1230	1240	1250	1260
5/P5	1280	1290	1300	1310	1320
C-DAF.seq					
RC of B/C DAF					

	GAAGAGTTAAGAAGAAAATACACACAAGTATACAGACTGTTCCCTAGTTCTTAGACTT				

	1280	1290	1300	1310	1320
5/P5	1280	1290	1300	1310	1320
C-DAF.seq					
RC of B/C DAF					

	ATCTGCATATTGGATAAAATAAATGCAATTGTGCTCTTCATTAGGATGCTTTCATTG				

Fig. 9 (part 7 of 11)

16/23

5/P5
C-DAF.seq
RC of B/C DAF
1340 1350 1360 1370 1380 1390
| | | | |

TCTTAAGATGTGTAGGAATGTCAACAGAGCAAGGAGAAAAAGGCAGTCCTGGAAT

5/P5
C-DAF.seq
RC of B/C DAF
1400 1410 1420 1430 1440 1450
| | | | |

CACATTCTTAGCACACCTACACCTCTTGAAAATAGAACAACTTGCAGAAATTGAGAGTG

5/P5
C-DAF.seq
RC of B/C DAF
1460 1470 1480 1490 1500
| | | | |

ATTCCTTCCCTAAAGTGTAAGAAAGCATAGAGATTGTTCGTATTAGAAATGGGATC

Fig. 9 (part 8 of 11)

5/P5	1510	1520	1530	1540	1550	1560
C-DAF.seq						
RC of B/C DAF						
5/P5	1570	1580	1590	1600	1610	1620
C-DAF.seq						
RC of B/C DAF						
5/P5	1630	1640	1650	1660	1670	1680
C-DAF.seq						
RC of B/C DAF						
5/P5	1690	1700	1710	1720	1730	1740
C-DAF.seq						
RC of B/C DAF						

Fig. 9 (part 9 of 11)

5/P5 C-DAF.seq RC of B/C DAF	1750	1760	1770	1780	1790

	AAACATGCTGCTGAACCAAGGGTGTGATGGTGATAAGGGAGGAATATAGAATGAAAG				
5/P5 C-DAF.seq RC of B/C DAF	1800	1810	1820	1830	1840

	ACTGAATCTTCCTTTGTTGCACAAATAGAGTTTGGAAGCCCTGTGAAAGGTGCTTT				
5/P5 C-DAF.seq RC of B/C DAF	1860	1870	1880	1890	1900

	CTTGACTTAATGTCTTTAAAGTATCCAGAGATACTACAATTAACATAAGAAAG				
5/P5 C-DAF.seq RC of B/C DAF	1920	1930	1940	1950	1960

	ATTATATTATTCTGAATCGAGATGTCCATAGTCAAATTGTAAATCTTATTT				

Fig. 9 (part 10 of 11)

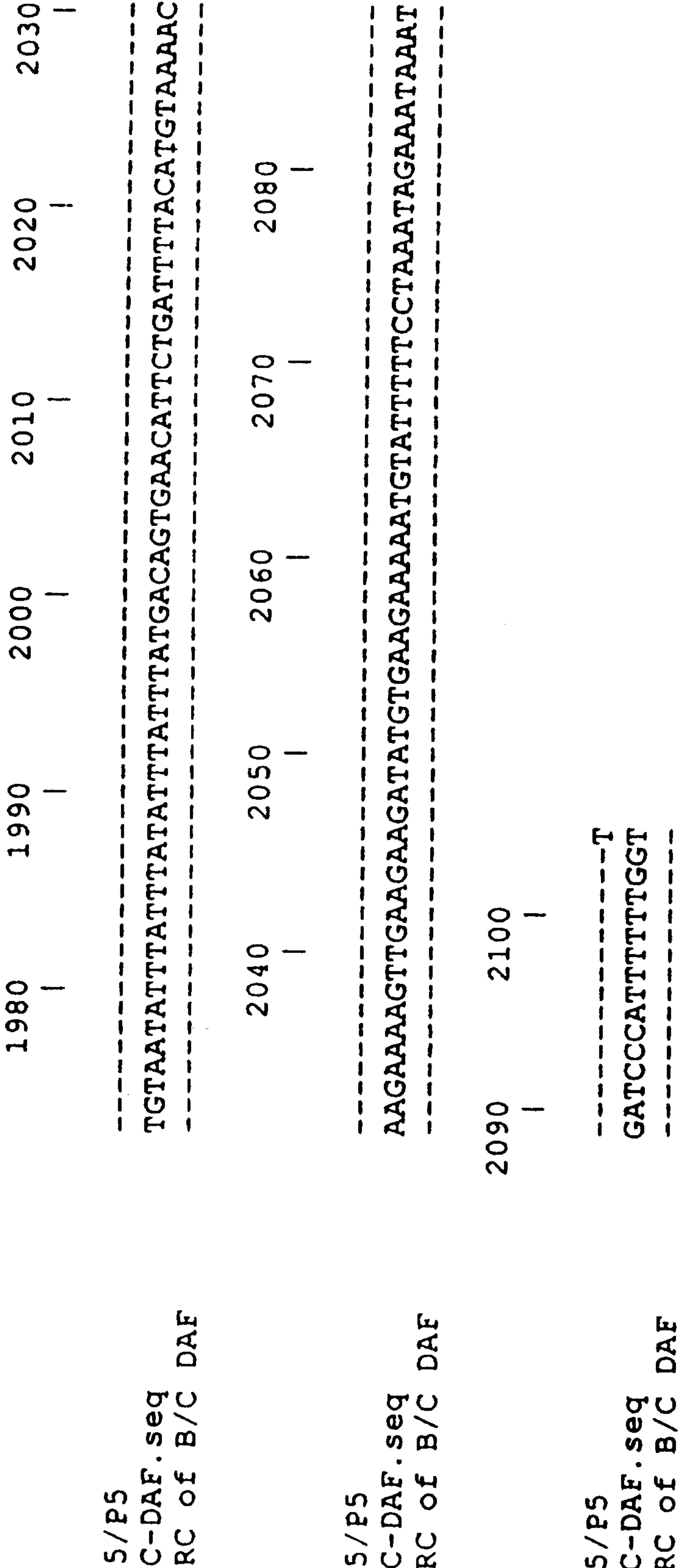


Fig. 9 (part 11 of 11)

20/23

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1                                     CC GCT GGG CGT AGC
15  TGC GAC TCG GCG GAG TCC CGG CGG CGC GTC CTT GTT CTA ACC CGG CGC GCC
    |<-- Leader sequence
    Met Thr Val Ala Arg Pro Ser Val Pro Ala Ala Leu Pro Leu Leu Gly Glu 17
66  ATG ACC GTC GCG CGG CCG AGC GTG CCC GCG GCG CTG CCC CTC CTC GGG GAG
    Leu Pro Arg Leu Leu Leu Leu Val Leu Leu Cys Leu Pro Ala Val Trp Gly 34
117 CTG CCC CGG CTG CTG CTG CTG GTG CTG TTG TGC CTG CCG GCC GTG TGG GGT
    <- sushi 1
    |<-- mature sequence (first 16 amino acids sequenced)
    Asp Cys Gly Leu Pro Pro Asp Val Pro Asn Ala Gln Pro Ala Leu Glu Gly 51
168 GAC TGT GGC CTT CCC CCA GAT GTA CCT AAT GCC CAG CCA GCT TTG GAA GGC
    Arg Thr Ser Phe Pro Glu Asp Thr Val Ile Thr Tyr Lys Cys Glu Glu Ser 68
219 CGT ACA AGT TTT CCC GAG GAT ACT GTA ATA ACG TAC AAA TGT GAA GAA AGC
    Phe Val Lys Ile Pro Gly Glu Lys Asp Ser Val Ile Cys Leu Lys Gly Ser 85
270 TTT GTG AAA ATT CCT GGC GAG AAG GAC TCA GTG ATC TGC CTT AAG GGC AGT
    sushi 1 ->| |sushi 2
    Gln Trp Ser Asp Ile Glu Glu Phe Cys Asn Arg Ser Cys Glu Val Pro Thr 102
321 CAA TGG TCA GAT ATT GAA GAG TTC TGC AAT CGT AGC TGC GAG GTG CCA ACA
    Arg Leu Asn Ser Ala Ser Leu Lys Gln Pro Tyr Ile Thr Gln Asn Tyr Phe 119
372 AGG CTA AAT TCT GCA TCC CTC AAA CAG CCT TAT ATC ACT CAG AAT TAT TTT
    Pro Val Gly Thr Val Val Glu Tyr Glu Cys Arg Pro Gly Tyr Arg Arg Glu 136
423 CCA GTC GGT ACT GTT GTG GAA TAT GAG TGC CGT CCA GGT TAC AGA AGA GAA
    Pro Ser Leu Ser Pro Lys Leu Thr Cys Leu Gln Asn Leu Lys Trp Ser Thr 153
474 CCT TCT CTA TCA CCA AAA CTA ACT TGC CTT CAG AAT TTA AAA TGG TCC ACA
    sushi 2->| |<- sushi 3
    Ala Val Glu Phe Cys Lys Lys Lys Ser Cys Pro Asn Pro Gly Glu Ile Arg 170
525 GCA GTC GAA TTT TGT AAA AAG AAA TCA TGC CCT AAT CCG GGA GAA ATA CGA
    Asn Gly Gln Ile Asp Val Pro Gly Gly Ile Leu Phe Gly Ala Thr Ile Ser 187
576 AAT GGT CAG ATT GAT GTA CCA GGT GGC ATA TTA TTT GGT GCA ACC ATC TCC
    Phe Ser Cys Asn Thr Gly Tyr Lys Leu Phe Gly Ser Thr Ser Ser Phe Cys 204
627 TTC TCA TGT AAC ACA GGG TAC AAA TTA TTT GGC TCG ACT TCT AGT TTT TGT
    sushi 3 ->|
    Leu Ile Ser Gly Ser Ser Val Gln Trp Ser Asp Pro Leu Pro Glu Cys Arg 221
678 CTT ATT TCA GGC AGC TCT GTC CAG TGG AGT GAC CCG TTG CCA GAG TGC AGA
    |<- sushi 4
    Glu Ile Tyr Cys Pro Ala Pro Pro Gln Ile Asp Asn Gly Ile Ile Gln Gly 238
729 GAA ATT TAT TGT CCA GCA CCA CCA CAA ATT GAC AAT GGA ATA ATT CAA GGG

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Fig. 10 (part 1 of 2)

21/23

780	Glu Arg Asp His Tyr Gly Tyr Arg Gln Ser Val Thr Tyr Ala Cys Asn Lys	255
	GAA CGT GAC CAT TAT GGA TAT AGA CAG TCT GTA ACG TAT GCA TGT AAT AAA	
831	Gly Phe Thr Met Ile Gly Glu His Ser Ile Tyr Cys Thr Val Asn Asn Asp	272
	GGA TTC ACC ATG ATT GGA GAG CAC TCT ATT TAT TGT ACT GTG AAT AAT GAT	
	sushi 4->	
885	Glu Gly Glu Trp Ser Gly Pro Pro Pro Glu Cys Arg Gly Lys Ser Leu Thr	289
	GAA GGA GAG TGG AGT GGC CCA CCA CCT GAA TGC AGA GGA AAA TCT CTA ACT	
933	Ser Lys Val Pro Pro Thr Val Gln Lys Pro Thr Thr Val Asn Val Pro Thr	306
	TCC AAG GTC CCA CCA ACA GTT CAG AAA CCT ACC ACA GTA AAT GTT CCA ACT	
984	Thr Glu Val Ser Pro Thr Ser Gln Lys Thr Thr Thr Lys Thr Thr Thr Pro	323
	ACA GAA GTC TCA CCA ACT TCT CAG AAA ACC ACC ACA AAA ACC ACC ACA CCA	
1035	Asn Ala Gln Ala Thr Arg Ser Thr Pro Val Ser Arg Thr Thr Lys His Phe	340
	AAT GCT CAA GCA ACA CGG AGT ACA CCT GTT TCC AGG ACA ACC AAG CAT TTT	
1086	His Glu Thr Thr Pro Asn Lys Gly Ser Gly Thr Thr Ser Gly Thr Thr Arg	357
	CAT GAA ACA ACC CCA AAT AAA GGA AGT GGA ACC ACT TCA GGT ACT ACC CGT	
1137	Leu Leu Ser Gly His Thr Cys Phe Thr Leu Thr Gly Leu Leu Gly Thr Leu	374
	CTT CTA TCT GGG CAC ACG TGT TTC ACG TTG ACA GGT TTG CTT GGG ACG CTA	
1188	Val Thr Met Gly Leu Leu Thr Stop	381
	GTA ACC ATG GGC TTG CTG ACT TAG CCAAAGAAGAGTTAAGAAG	
1231	AAAATACACACAAGTATACAGACTGTTTCCTAGTTTCTTAGACTTATCTGCATATTGGATAAAATAAA	
1298	TGCAATTGTGCTCTTCATTTAGGATGCTTTTCATTGTCTTTAAGATGTGTTAGGAATGTCAACAGAGC	
1365	AAGGAGAAAAAAGGCAGTCCTGGAATCACATTCTTAGCACACCTACACCTCTTGAAAATAGAACAAC	
1432	TTGCAGAATTGAGAGTGATTCCCTTTCCTAAAGTGTAAGAAAGCATAGAGATTTGTTCCGTATTTAGA	
1499	ATGGGATCACGAGGAAAAGAGAAGGAAAGTGATTTTTTTCCACAAGATCTGTAATGTTATTTCCACT	
1566	TATAAAGGAAATAAAAAATGAAAAACATTATTTGGATATCAAAAGCAAATAAAAACCCAATTCAGTC	
1633	TCTTCTAAGCAAAATTGCTAAAGAGAGATGAACCACATTATAAAGTAATCTTTGGCTGTAAGGCATT	
1700	TTCATCTTTTCCTTCGGGTTGGCAAATATTTTAAAGGTAAAACATGCTGGTGAACCAGGGGTGTTGA	
1767	TGGTGATAAGGGAGGAATATAGAATGAAAGACTGAATCTTCCTTTGTTGCACAAATAGAGTTTGGAA	
1834	AAAGCCTGTGAAAGGTGTCTTCTTTGACTTAATGTCTTTAAAGTATCCAGAGATACTACAATATTA	
1901	ACATAAGAAAAGATTATATATTATTTCTGAATCGAGATGTCCATAGTCAAATTTGTAAATCTTATTC	
1968	TTTTGTAATATTTATTTATATTTATTTATGACAGTGAACATTCTGATTTTACATGTAAAACAAGAAA	
2035	AGTTGAAGAAGATATGTGAAGAAAAATGTATTTTTCCTAAATAGAAATAAATGATCCCATTTTTTGG	
2102	T	

Fig. 10 (part 2 of 2)

22/23

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1      TTTAAACGGGCCCTCTAGACTCGAGCGGCCGCTGCCCATCTTGTCGTCGTCGTCCTTGTAGTCG
65    TGCATGTGGTGGTGGTGGTGGTGGTTAACCATGGTGGCGGGCCGCCACTGTGCTGGATATCTGCAGA
132   ATTCGATGGGCGTAGCTGCGACTCGGCGGAGTCCCGGCGGCGCGTCCTTGTCTAACCCGGCGCGCC

      Met Thr Val Ala Arg Pro Ser Val Pro Ala Ala Leu Pro Leu Leu Gly Glu  17
199   ATG ACC GTC GCG CGG CCG AGC GTG CCC GCG GCG CTG CCC CTC CTC GGG GAG

      Leu Pro Arg Leu Leu Leu Leu Val Leu Leu Cys Leu Pro Ala Val Trp Gly  34
250   CTG CCC CGG CTG CTG CTG CTG GTG CTG TTG TGC CTG CCG GCC GTG TGG GGT

      Asp Cys Gly Leu Pro Pro Asp Val Pro Asn Ala Gln Pro Ala Leu Glu Gly  51
301   GAC TGT GGC CTT CCC CCA GAT GTA CCT AAT GCC CAG CCA GCT TTG GAA GGC

      Arg Thr Ser Phe Pro Glu Asp Thr Val Ile Thr Tyr Lys Cys Glu Glu Ser  68
352   CGT ACA AGT TTT CCC GAG GAT ACT GTA ATA ACG TAC AAA TGT GAA GAA AGC

      Phe Val Lys Ile Pro Gly Glu Lys Asp Ser Val Ile Cys Leu Lys Gly Ser  85
403   TTT GTG AAA ATT CCT GGC GAG AAG GAC TCA GTG ATC TGC CTT AAG GGC AGT

      Gln Trp Ser Asp Ile Glu Glu Phe Cys Asn Arg Ser Cys Glu Val Pro Thr 102
454   CAA TGG TCA GAT ATT GAA GAG TTC TGC AAT CGT AGC TGC GAG GTG CCA ACA

      Arg Leu Asn Ser Ala Ser Leu Lys Gln Pro Tyr Ile Thr Gln Asn Tyr Phe 119
505   AGG CTA AAT TCT GCA TCC CTC AAA CAG CCT TAT ATC ACT CAG AAT TAT TTT

      Pro Val Gly Thr Val Val Glu Tyr Glu Cys Arg Pro Gly Tyr Arg Arg Glu 136
556   CCA GTC GGT ACT GTT GTG GAA TAT GAG TGC CGT CCA GGT TAC AGA AGA GAA

      Pro Ser Leu Ser Pro Lys Leu Thr Cys Leu Gln Asn Leu Lys Trp Ser Thr 153
607   CCT TCT CTA TCA CCA AAA CTA ACT TGC CTT CAG AAT TTA AAA TGG TCC ACA

      Ala Val Glu Phe Cys Lys Lys Lys Ser Cys Pro Asn Pro Gly Glu Ile Arg 170
658   GCA GTC GAA TTT TGT AAA AAG AAA TCA TGC CCT AAT CCG GGA GAA ATA CGA

      Asn Gly Gln Ile Asp Val Pro Gly Gly Ile Leu Phe Gly Ala Thr Ile Ser 187
709   AAT GGT CAG ATT GAT GTA CCA GGT GGC ATA TTA TTT GGT GCA ACC ATC TCC

      Phe Ser Cys Asn Thr Gly Tyr Lys Leu Phe Gly Ser Thr Ser Ser Phe Cys 204
760   TTC TCA TGT AAC ACA GGG TAC AAA TTA TTT GGC TCG ACT TCT AGT TTT TGT

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Fig. 11 (part 1 of 2)

23/23

Leu Ile Ser Gly Ser Ser Val Gln Trp Ser Asp Pro Leu Pro Glu Cys Arg 221
 811 CTT ATT TCA GGC AGC TCT GTC CAG TGG AGT GAC CCG TTG CCA GAG TGC AGA

Glu Ile Tyr Cys Pro Ala Pro Pro Gln Ile Asp Asn Gly Ile Ile Gln Gly 238
 862 GAA ATT TAT TGT CCA GCA CCA CCA CAA ATT GAC AAT GGA ATA ATT CAA GGG

Glu Arg Asp His Tyr Gly Tyr Arg Gln Ser Val Thr Tyr Ala Cys Asn Lys 255
 913 GAA CGT GAC CAT TAT GGA TAT AGA CAG TCT GTA ACG TAT GCA TGT AAT AAA

Gly Phe Thr Met Ile Gly Glu His Ser Ile Tyr Cys Thr Val Asn Asn Asp 272
 964 GGA TTC ACC ATG ATT GGA GAG CAC TCT ATT TAT TGT ACT GTG AAT AAT GAT

Glu Gly Glu Trp Ser Gly Pro Pro Pro Glu Cys Arg Gly Lys Ser Leu Thr 289
 1015GAA GGA GAG TGG AGT GGC CCA CCA CCT GAA TGC AGA GGA AAA TCT CTA ACT

Ser Lys Val Pro Pro Thr Val Gln Lys Pro Thr Thr Val Asn Val Pro Thr 306
 1066TCC AAG GTC CCA CCA ACA GTT CAG AAA CCT ACC ACA GTA AAT GTT CCA ACT

Thr Glu Val Ser Pro Thr Ser Gln Lys Thr Thr Thr Lys Thr Thr Thr Pro 323
 1117ACA GAA GTC TCA CCA ACT TCT CAG AAA ACC ACC ACA AAA ACC ACC ACA CCA

Asn Ala Gln Ala Thr Arg Ser Thr Pro Val Ser Arg Thr Thr Lys His Phe 340
 1168AAT GCT CAA GCA ACA CGG AGT ACA CCT GTT TCC AGG ACA ACC AAG CAT TTT

His Glu Thr Thr Pro Asn Lys Gly Ser Gly Thr Thr Ser Gly Thr Thr Arg 357
 1219CAT GAA ACA ACC CCA AAT AAA GGA AGT GGA ACC ACT TCA GGT ACT ACC CGT

Leu Leu Ser Gly His Thr Cys Phe Thr Leu Thr Gly Leu Leu Gly Thr Leu 374
 1270CTT CTA TCT GGG CAC ACG TGT TTC ACG TTG ACA GGT TTG CTT GGG ACG CTA

Val Thr Met Gly Leu Leu Thr Stop
 1321GTA ACC ATG GGC TTG CTG ACT TAG CCAAAGAAGAGTTAAGAAGAAAATACACACAAGTA 381

1380TACAGACTGTTCTAGTTTCTTAGACTTATCTGCATATTGGATAAAATAAATGCAATTGTGCTCTTC
 1447ATTTAGGATGCTTTCATTGTCTTTAAGATGTGTTAGGAATGTCAACA

Fig. 11 (part 2 of 2)

#2321974

UNSCANNABLE ITEM

RECEIVED WITH THIS APPLICATION

(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

DOCUMENT REÇU AVEC CETTE DEMANDE

NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈME ÉTAGE AIRE 5 DANS LA SECTION DE LA
PRÉPARATION DES DOSSIERS)