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The α Subunit of the GTP Binding Protein G_k Opens Atrial Potassium Channels

JUAN CODINA, ATSUKO YATANI, DAGOBERTO GRENET, ARTHUR M. BROWN, LUTZ BIRNBAUMER

Guanine nucleotide binding (G) proteins (subunit composition αβγ) dissociate on activation with guanosine triphosphate (GTP) analogs and magnesium to give α–guanine nucleotide complexes and free βγ subunits. Whether the opening of potassium channels by the recently described G_k in isolated membrane patches from mammalian atrial myocytes was mediated by the α_k subunit or βγ dimer was tested. The α_k subunit was found to be active, while the βγ dimer was inactive in stimulating potassium channel activity. Thus, G_k resembles G_s, the stimulatory regulatory component of adenylyl cyclase, and transducin, the regulatory component of the visual system, in that it regulates its effector function—the activity of the ligand-gated potassium channel—through its guanine nucleotide binding subunit.

RECENT WORK (*1, 2*) DEMONSTRATED that a heterotrimeric pertussis toxin (PTX)–sensitive guanine nucleotide binding (G) protein, purified from human red blood cells (hRBC) and treated with guanosine (γ-thio)triphosphate (GTPγS) and magnesium, causes opening of a subset of potassium channels present in isolated membrane patches of atrial cells from adult guinea pig hearts and pituitary GH_3 tumor cells. The properties of these channels are similar to those activated in these cells by receptor ligands such as acetylcholine (ACh), acting through muscarinic receptors (mAChR), and somatostatin, acting through its separate specific receptors. The channels are also opened by GTPγS and Mg^{2+}, which presumably act by stimulating a G protein endogenous to the cardiac membrane patch. Based on its activity, we called this G protein G_k (*3*).

The response to receptor ligands is guanosine triphosphate (GTP)–dependent and abolished by PTX, and, after PTX treatment, is restored by addition of untreated G_k from hRBC in the presence of GTP. Thus, ligand-induced opening of K^+ channels in isolated membrane patches is controlled in a manner analogous to hormonal stimulation of adenylyl cyclases; G_k activation is GTP-dependent and stimulated by occupied receptors, and K^+ channels are the effectors "sensing" the activity state of G_k.

In our experiments (*1, 2*), opening of K^+ channels was specific for the PTX-sensitive G protein of hRBC and occurred at picomolar concentrations. G_s at nanomolar concentrations was unable to substitute for G_k either in coupling receptors to the K^+ channel in the presence of GTP or, after treatment with GTPγS and Mg^{2+}, in directly causing K^+ channel opening.

It is commonly thought that activation of G proteins involves tight binding of the guanine nucleotide (a Mg^{2+}-dependent step) and dissociation of the heterotrimer into an activated α–G nucleotide complex plus free βγ. The purified α_s-GTPγS complex is sufficient for stimulation of adenylyl cyclase (*4*). For inhibition of adenylyl cyclase, the situation is more complex because, even though resolved α_i-GTPγS complexes purified from liver were shown to inhibit enzyme activity by Katada *et al.* (*5*), βγ dimers also inhibited adenylyl cyclase and did so at lower concentrations. Although they did not discount a role for α_i, these authors proposed that βγ dimers may account for a major proportion of G_i-mediated inhibition of adenylyl cyclase (*5*).

Human erythrocyte G_k, with which we stimulated K^+ channels and reconstituted receptors coupling to K^+ channels after treatment with PTX, is also formed of αβγ subunits and dissociates into α-GTPγS plus βγ under the conditions used for activation and testing of its activity (*6*). Because GTPγS-treated G_s, a mixture of α_s-GTPγS plus βγ subunits that are biochemically indistinguishable from those present in the human erythrocyte G_k (*7*), did not stimulate K^+ channels, it seemed that the α subunit and not the βγ dimer was responsible for the effect of G_k and K^+ channels.

Unexpectedly, however, Logothetis *et al.* (*8*) reported opening of atrial K^+ channels in isolated membrane patches from embryonic chick atria to be caused by βγ complexes and not by α subunits purified from bovine brain (*9, 10*). The bovine brain βγ preparations had previously been shown by two-dimensional tryptic peptide mapping to be indistinguishable from human erythrocyte βγ dimers (*7*).

In light of these conflicting results, we reexamined the validity of our inferred conclusion that α_k, rather than βγ from G_k, caused the K^+ channel opening we had observed. We prepared βγ complexes, resolved as well as possible from PTX-sensitive α subunits, and α_k-GTPγS complexes, resolved as well as possible from βγ subunits (Fig. 1), and tested their individual effects on guinea pig atrial K^+ channels as described (*1*).

Ion exchange chromatography (*11*) was used to obtain α_k-GTPγS (α_k*) from GTPγS-activated human erythrocyte G_k (G_k*). The resulting preparation was analyzed by SDS–polyacrylamide gel electrophoresis followed by densitometry of the gel after staining first with Coomassie blue and then with silver; the standards were increasing amounts of bovine serum albumin ranging from 0.001 to 10 μg per lane. We obtained a solution that contained 36 μg of α_k* per milliliter and 0.4 μg of βγ per milliliter, to which we ascribed a nominal concentration of 1 μ*M* α_k* and 0.01 μ*M* βγ (estimation error, ±10%). The Coomassie blue–stained polyacrylamide gel onto which 2 μg of the starting hRBC G_k and 0.5 μg of the resulting hRBC α_k* had been applied is shown in Fig. 1. The method used to prepare α_k* did not yield significant amounts of free βγ subunits. These were prepared separately from human erythrocytes (Fig. 1) and bovine brain (Fig. 1) with methods that do not involve stabilization with activating

J. Codina and D. Grenet, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.
A. Yatani and A. M. Brown, Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.
L. Birnbaumer, Department of Cell Biology and Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.

ligands such as NaF and $AlCl_3$ and avoid the use of Mg^{2+} ions (*12*).

In view of the report of Logothetis *et al.* (*8*), we first investigated the possible functional effects of βγ preparations from bovine brain, that is, of subunits of the same origin as theirs. The muscarinic K^+ channels of interest can be unambiguously identified in mammalian atrial cells by their conductance, open time duration, and inward rectification (*1, 13*). We observed no effect of 2 to 4 n*M* untreated βγ on the guinea pig atrial muscarinic K^+ channels (six experiments). We also observed no effect of 2 to 4 n*M* untreated βγ on channels in patches held with pipettes containing 10 μ*M* carbachol and incubated with PTX and nicotinamide adenine dinucleotide (NAD^+) until the coupling activity of the endogenous G_k had been eliminated (four experiments). However, significant K^+ channel opening was consistently obtained on addition of 100 to 200 p*M* of the bovine brain βγ preparation after treating it with GTPγS and Mg^{2+}, followed by dialysis, in the same way as G_k was treated to prepare G_k* (four experiments). This "βγ*" preparation at 100 to 200 p*M* produced far less frequent openings than 50 to 100 p*M* G_k*, suggesting that the effect might be due to contaminating G_k. Indeed, even though analysis by SDS–polyacrylamide gel electrophoresis followed by Coomassie blue staining had shown the bovine brain βγ to be "pure" (Fig. 1), analysis for contaminating G_k by adenosine diphosphate (ADP)–ribosylation with PTX and [^{32}P]NAD^+ readily revealed presence of approximately 3% G_k in our preparation of bovine brain βγ (Fig. 2A). A similar test for the presence of a PTX-sensitive holo–G protein in preparations of human erythrocyte βγ was negative (Fig. 2B). We next tested resolved α_k* and resolved βγ from human erythrocytes for their capability to stimulate K^+ channels.

In contrast to the report of Logothetis *et al.* (*8*), GTPγS-complexed α subunits of the PTX-sensitive G protein—actual molar ratio of α to βγ of about 100:1—were very potent stimulators of K^+ channel activity. Previous experiments had shown K^+ channel opening to appear on addition of 0.2 to 1 p*M* GTPγS-treated holo-G_k (G_k*) (*1*). In our present studies (Fig. 3A) (representative of 20 similar experiments), α_k* was equipotent on a molar basis with that of the starting G_k*. We observed K^+ channel opening in three out of seven trials after addition of 0.5 p*M* α_k-GTPγS (threshold concentration). Frequent openings, occurring in bursts and clusters of bursts (*14*), were obtained in 14 out of 14 trials on addition of 5 p*M* α_k*. On addition of 50 p*M* α_k* even more frequent openings were obtained, including simultaneous openings of more than one of the channels in the patch. In all instances, α_k* was tested on membrane patches that in their inside-out configuration had shown no opening for at least 5 minutes.

The unitary openings induced by α_k* and G_k* were the same within the error of the measurements. At −80 mV, the unitary currents had amplitudes of 2.18 ± 0.19 pA (mean ± SD from four separate experiments) when induced by α_k* and 2.13 ± 0.11 pA (n = 6) when induced by G_k*. Similarly, the mean open times of channels stimulated by α_k* (1.23 ± 0.24 msec; n = 4) did not differ significantly from those of channels stimulated by G_k* (1.15 ± 0.25 msec; n = 6). We concluded that preparations of G_k* and α_k* caused opening of the same K^+ channels.

Further emphasis on the specificity of the α subunits came from the findings that βγ preparations from human erythrocytes (Figs. 1 and 3B) were inactive at 2 to 4 n*M* in stimulating K^+ channels. This was regardless of whether the βγ preparations had been incubated with GTPγS under G_k activating conditions (Fig. 3B, representative of five such experiments; Fig. 3C, representative of four such experiments).

In these experiments, we noticed that prior exposure of patches to high concentrations (2 to 4 n*M*) of βγ often delayed the stimulation of K^+ channels by G_k* or α_k*. K^+ channel opening in such patches never occurred within the first 2 minutes after

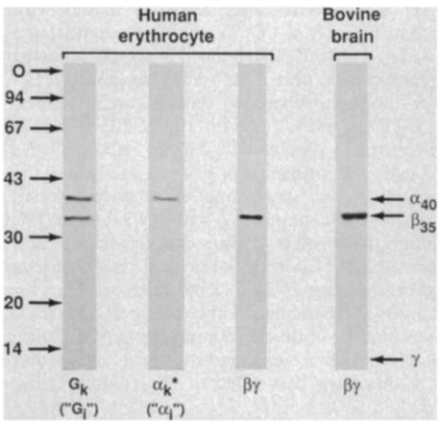

Fig. 1. SDS–polyacrylamide gel electrophoresis (10% acrylamide) of preparations used in our studies: G_K (2 μg holo–G protein), α_k-GTPγS (α_k*) (0.5 μg protein), βγ dimers (1.0 μg of protein) from human erythrocytes, and βγ dimers (2 μg of protein) from bovine brain. Bovine brain membranes were prepared according to Neer *et al.* (*9*). G_k and βγ dimers were prepared from erythrocyte and bovine brain membranes according to Codina *et al.* (*12*), and stored at −70°C in 0.1% Lubrol-PX, 1 m*M* EDTA, 20 m*M* β-mercaptoethanol, 150 m*M* NaCl, 10 m*M* sodium Hepes, *p*H 8.0, and 30% (by volume) ethylene glycol (buffer A) at 100 to 200 μg/ml. α_k* was prepared as follows: 750 μl of G_k (106 μg/ml) in buffer A with 5% Lubrol-PX, 200 μ*M* GTPγS, and 100 m*M* $MgCl_2$ were incubated for 60 minutes at 32°C, diluted 13-fold with 1 m*M* dithiothreitol (DTT), 1 m*M* EDTA, 10 m*M* tris-HCl, *p*H 8.0, and 0.6% Lubrol-PX (buffer B). This solution was applied to a column of DEAE-Fractogel TSK 650M (Pierce) of 0.1-ml bed volume equilibrated with buffer B. The column was washed sequentially with 2 ml of 7 m*M* $MgCl_2$, 1 m*M* DTT, 1 m*M* EDTA, and 10 m*M* tris-HCl, *p*H 8.0, (buffer C) and 1.0 ml of buffer C plus 60 m*M* NaCl. α_k* was eluted with a final yield of 22% in two 0.25-ml aliquots of buffer C containing 200 m*M* NaCl. These fractions were dialyzed for 12 hours with three changes of 650 ml of buffer C with 20 m*M* KCl. The figure is a composite of three Coomassie blue–stained gels; G_k and α_k*—also called "G_i" and "α_i*" (*1, 3*)—were electrophoresed on the same gel. γ subunits, which migrate with the dye front, are not visible because of their poor staining properties (*7*).

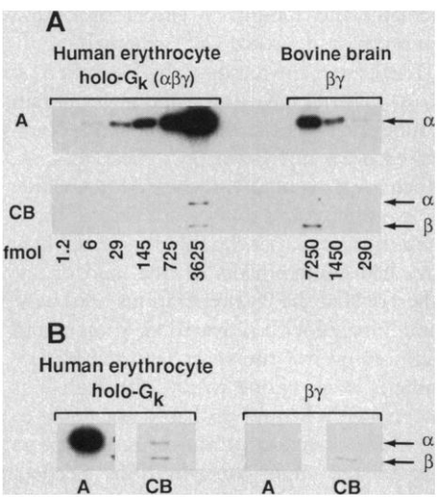

Fig. 2. Analysis of presence of PTX substrate in (**A**) bovine brain and (**B**) human erythrocyte βγ preparations used in our studies. Photographs of the autoradiographs (A) and of the stained gels (CB) of the regions where α and β subunits migrate are shown; γ subunits migrated with the dye front and are not shown. [^{32}P]ADP-ribosylation of increasing quantities of G_k, used as standards, and the indicated amounts of the bovine brain βγ preparation shown in Fig. 1. [^{32}P]-ADP-ribosylation of 5000 fmol each of G_k (400 ng) and human erythrocyte βγ (200 ng). Dialyzed PTX (*12*) was incubated at 300 μg/ml with 20 m*M* DTT for 20 minutes at 32°C, diluted fivefold with 0.4% bovine serum albumin to give activated PTX. Fractions (10 μl) to be covalently modified with adenosine diphosphoribose (ADP-ribose) were incubated in a final volume of 60 μl containing 10 μl activated PTX, 1 m*M* adenosine triphosphate, 100 μ*M* GTP, 1 m*M* EDTA, 10 m*M* tris-HCl, *p*H 7.6, 0.25% Lubrol-PX, and 10 μ*M* [^{32}P]NAD^+. Proteins in the reaction mixtures were precipitated with acetone, washed with 15% ice cold trichloroacetic acid and ether, and electrophoresed on 10% SDS-polyacrylamide gels (*12*). The gel slabs were stained with Coomassie blue, photographed, dried under vacuum, and autoradiographed for 16 hours.

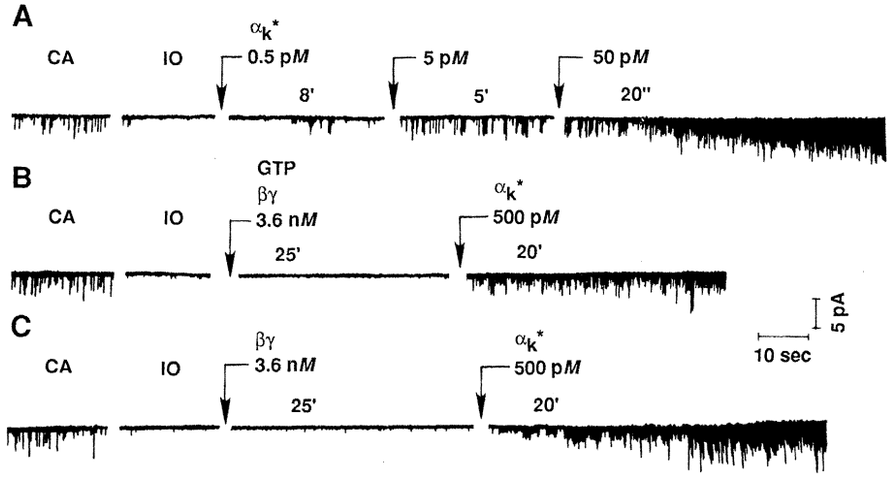

Fig. 3. Effects of (**A**) human erythrocyte α_k* and resolved (**B** and **C**) human erythrocyte βγ subunits in Figs. 1 and 2 on K^+ channel activity in excised inside-out membrane patches of adult guinea pig atrial cells. Atrial myocytes were prepared by enzymatic dispersion (*17*) and K^+ currents were recorded by patch-clamp techniques (*18*) in the cell-attached (CA) and excised inside-out (IO) modes with symmetrical isotonic K^+ (140 m*M*) solutions as described in Yatani *et al.* (*1*). The holding potential was −80 mV; pipette solutions were 140 m*M* KCl, 1 m*M* EGTA, 1 m*M* $MgCl_2$, 5 m*M* Hepes (*p*H adjusted to 7.3 with tris base); bathing solutions had the same composition plus 2 m*M* ATP [to inhibit ATP-sensitive K^+ currents (*19*)] and 100 μ*M* adenosine 3′,5′-monophosphate (to avoid secondary effects due to possible inhibition of adenylyl cyclase) without GTP (A and C) or with 100 μ*M* GTP added with βγ (B). Dilution of proteins: α_k* subunits were diluted in pipette solution; βγ subunits were diluted with buffer A (Fig. 1) to 80 to 160 μg/ml (2 to 4 μ*M*) and, immediately prior to addition to the experimental chamber, another 100-fold in ice-cold pipette buffer containing 0.1% bovine serum albumin; βγ* was prepared by incubation of βγ at 200 to 400 μg/ml in buffer A with 0.1% Lubrol-PX, 100 μ*M* GTPγS and 50 m*M* $MgCl_2$ at 32°C for 30 minutes, followed by dialysis as above for α_k* and dilution according to the scheme used for dilution of untreated βγ. Additions of proteins were made as 10-μl aliquots to a 100-μl experimental chamber placed on the stage of an inverted microscope. After addition, the chamber solution was rapidly mixed with a 20-μl Pipetteman. Times at top of each trace are in minutes (′) or seconds (″) elapsed between the preceding addition and the recording of the trace shown. The first additions were made between 7 and 10 minutes after excision of the patch, and further additions were made at 25- to 30-minute intervals. Calibration bars refer to all records. Other conditions were as in Yatani *et al.* (*1, 2*).

addition of G_k* or α_k* ($n = 15$) and in several instances ($n = 4$) took 20 or more minutes to appear. This was not due to the buffer added with the βγ subunits and was observed with βγ (untreated, GTP present) from both bovine brain and human erythrocytes. This suggested that βγ subunits may have an inhibitory activity. We tested for a potential inhibitory activity of the human erythrocyte βγ by adding it to membrane patches held by pipettes containing 10 μ*M* carbachol and in bathing media with 100 μ*M* GTP, that is, to patches with ligand-stimulated K^+ channels. In two out of five trials, we observed a decrease in K^+ channel activity, which was overcome on addition of α_k* or G_k*. These experiments suggest that free βγ subunits may play an inhibitory role, similar to that by which βγ subunits may lower adenylyl cyclase activity (*5*). However, it is cautioned that we have only used very high concentrations (three to four orders of magnitude higher than those at which α_k* is able to stimulate K^+ channels) and have not carried out sufficient experiments to ascribe any physiological role to these observations.

Our experiments demonstrate that of the two complexes that form when G_k is treated with GTPγS in the presence of Mg^{2+} to give G_k*, it is the α_k-GTPγS and not the βγ that cause opening of K^+ channels in guinea pig atrial myocytes. Recently Hescheler *et al.* (*15*) have shown that the α subunit of porcine brain G_o and not the βγ dimer mediates the inhibitory effects of opioid receptors on neuronal Ca^{2+} channels.

There are many reasons why Logothetis *et al.* (*8*) may not have found effects with brain α subunits but obtained K^+ channel opening with 23 n*M* brain βγ. These reasons, which are presented elsewhere (*16*), include the possibility that α_k was not among the α subunits tested, that the subunits tested may have had contaminants of one kind or another, or that the βγ preparations used were contaminated with activated G_k proteins. In addition, even if the effect reported by Logothetis *et al.* is due to βγ, although it is interesting, it would probably not relate to the mode of action of muscarinic receptors due to the high concentrations needed. More recently, Logothetis *et al.* showed their βγ preparation to be active at 200 p*M* and measured a response, which they indicated was quantitatively equivalent to ours at 200 p*M* G_k* (*16*). However, the response to bovine brain βγ shown (*16*) was at most 10% of that attainable in the same patch under the cell-attached configuration. In contrast, addition of 200 p*M* α_k* to the same membrane patch that is shown in Fig. 3A resulted in no further increase in activity than that seen with 50 p*M*. This indicates that our system may saturate at 50 p*M*. Thus, if patches were comparable, a 0.5% contamination of bovine brain βγ with activated G_k could still account for the results of Logothetis *et al.* (*8, 16*). Further, even if both α_k* and βγ are able to stimulate K^+ channels, α_k* does so at concentrations at least 100 times lower than βγ. Because α and βγ subunits are formed in equimolar amounts, the results indicate that the mechanism of action of G_k is similar to that of G_s and transducin in that its effector function is regulated through the α subunit.

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3. When originally purified, the human erythrocyte PTX-sensitive G protein was called "putative G_i (N_i)," later simplified to G_i (or N_i) (*12*). However, the identity between G_i activity in cells, which mediates hormonal inhibition of adenylyl cyclase, and this purified G protein was never established. Because the purified protein was recently found to be a potent activator of ligand-gated K^+ channels, we have renamed the human erythrocyte PTX-sensitive G protein G_k and its α subunit α_k, but also refer to it as "G_i" or "α_i," the quotes indicating our uncertainty as to its G_i or α_i activity. Abbreviations: G, guanine nucleotide binding signal transducing protein of composition αβγ, which is a stimulatory or inhibitory component of an effector system; G_s, stimulatory regulatory component of adenylyl cyclase; G_i, inhibitory regulatory component of adenylyl cyclase; G_k, stimulatory regulatory component of receptor-gated K^+ channels; α_s, α subunit of G_s; α_i, α subunit of G_i; α_k, α subunit of G_k; * denotes treatment of the protein or its subunit with GTPγS and Mg^{2+} under conditions that lead to dissociation of a G protein into an activated (*) α-GTPγS complex and a βγ dimer.
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Evidence for Increased Somatic Cell Mutations at the Glycophorin A Locus in Atomic Bomb Survivors

RICHARD G. LANGLOIS, WILLIAM L. BIGBEE, SEISHI KYOIZUMI, NORI NAKAMURA, MICHAEL A. BEAN, MITOSHI AKIYAMA, RONALD H. JENSEN*

A recently developed assay for somatic cell mutations was used to study survivors of the atomic bomb at Hiroshima. This assay measures the frequency of variant erythrocytes produced by erythroid precursor cells with mutations that result in a loss of gene expression at the polymorphic glycophorin A (GPA) locus. Significant linear relations between variant frequency (VF) and radiation exposure were observed for three different variant cell phenotypes. The spontaneous and induced VFs agree with previous measurements of radiation-induced mutagenesis in other systems; this evidence supports a mutational origin for variant cells characterized by a loss of GPA expression and suggests that the GPA assay system may provide a cumulative dosimeter of past radiation exposures. VFs for some survivors differ dramatically from the calculated dose response, and these deviations appear to result primarily from statistical fluctuations in the number of mutations in the stem-cell pool. These fluctuations allow one to estimate the number of long-lived hemopoietic stem cells in humans.

THERE IS CONTINUING INTEREST IN evaluating the long-term health effects of human exposure to ionizing radiation (*1*) and in developing methods for assessing genetic damage in exposed individuals. The survivors of the atomic bombs at Hiroshima and Nagasaki provide a population particularly well suited for quantitative studies of this type. Researchers at the Radiation Effects Research Foundation (RERF) in Japan are accumulating extensive clinical and experimental data on large numbers of exposed and control survivors and their progeny (*2*). The exposure history of survivors is relatively well defined; dose estimates are available for individual survivors, and their exposure is dominated by a single, acute, whole-body, radiation dose. Previous studies of chromosome aberrations have revealed persistent genetic damage in somatic cells of these individuals (*3*). This study of specific locus mutations in somatic cells should further enhance our understanding of radiation-induced health effects.

The present study uses a newly developed specific locus mutation assay to detect the loss of gene expression at the glycophorin A locus in human somatic cells (*4*). Glycophorin A (GPA), a cell-surface glycoprotein on erythrocytes, occurs in two allelic forms, M and N, and is the product of codominantly expressed alleles on chromosome 4. In the GPA expression-loss assay, pairs of monoclonal antibodies specific for individual allelic forms are each conjugated with a different fluorescent dye and used to label fixed erythrocytes from heterozygous MN donors. Flow cytometry and sorting are used to enumerate and purify rare, single-color cells that lack the expression of one of the two GPA alleles. Presumably these cells lack expression because they are progeny of mutated erythroid precursor cells. Two of three previously described assay systems [1W1 and 2W2 (*4*)] were used in this study to determine the frequency of four variant cell types: those that lack the M-form of GPA (hemizygous NØ and homozygous NN variants) and those that lack the N-form of GPA (MØ and MM variants).

Blood samples were obtained from heterozygous MN donors in the RERF Adult Health Study and from a standard donor. One cohort of 43 donors (exposed) was composed of Hiroshima A-bomb survivors with revised tentative 1965 radiation dose estimates (T65DR) (*5*) ranging from 14 to 884 cGy (1 cGy = 1 rad). Repeat samples were obtained from 27 of these donors. A second cohort of 20 donors (control), with age and sex ratio similar to the exposed cohort, was composed of Hiroshima survivors with T65DR dose estimates of less than 1 cGy, with repeat samples from 10 donors. The standard donor (standard), a 29-year-old Japanese volunteer, was sampled 27 times during the study to monitor assay reproducibility. All samples were coded and then analyzed blind within 2 weeks of collection.

Variant frequencies (VFs) from all measurements for both assay systems are shown in Fig. 1; the mean VFs for each cohort are given in Table 1. Consistently low VFs were observed for three of the four variant cell types (NØ, MØ, and MM) in samples from the standard and control donors. The mean VFs for these donors ranged from 7×10^{-6} to 15×10^{-6}, which agreed with previously determined values of 9×10^{-6} to 21×10^{-6} for normal donors (*4*). From multiple measurements of the standard donor and replicate measurements on repeat blood samples from control and exposed donors, we determined a measurement coefficient of variation (CV) of about 60%, which was also comparable to previous estimates. The scattered VFs for NN variants were probably caused by staining artifacts previously described for this cell type (*4*).

Among the exposed donors, VFs of the NØ, MØ, and MM cell types varied widely from control levels to a maximum of 1312×10^{-6}. We confirmed the significance of the differences between the control and exposed donors with the nonparametric Mann-Whitney test (*6*) (Table 1). The mean frequencies of NØ and MØ variants were similar for the exposed donors, data that suggest that radiation-induced loss of GPA expression occurs at comparable frequencies for both the M and N alleles. The elevated VF of MM variants in exposed donors suggests that radiation induces events that lead to homozygosity [for example, chromosome missegregation or mitotic recombination (*4*)], although these events appear to be induced at a lower level than gene-loss events. Radiation-induced missegregation or recombination events have been reported for yeast (*7*), *Drosophila* (*8*), and mammalian cells (*9, 10*).

To relate GPA-assayed VFs to each individual's estimated dose, we assumed that T65DR doses approximate bone marrow doses since current revisions in exposure and organ doses are not yet available (*11*). Because the data set is too small to allow a rigorous test of a linear versus quadratic dose response, we also assumed a linear

R. G. Langlois, W. L. Bigbee, R. H. Jensen, Biomedical Sciences Division, Lawrence Livermore National Laboratory, University of California, Livermore, CA 94550.
S. Kyoizumi, N. Nakamura, M. Akiyama, Radiation Effects Research Foundation, Hiroshima City 730, Japan.
M. A. Bean, Virginia Mason Research Center, Seattle, WA 98101.

*To whom correspondence should be addressed.