Expression and secretion of aequorin as a chimeric antibody by means of a mammalian expression vector

(antibody fusion/bioluminescence/cloning/recombinant DNA)

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ABSTRACT  A fusion protein has been expressed from the relevant genes in mammalian cells consisting of the photoprotein aequorin and an anti-4-hydroxy-3-nitrophenacetyl antibody gene. This chimeric antibody has allowed the development of a sensitive luminescent immunoassay. Initially the cDNA of the photoprotein aequorin from Aequorea victoria was cloned and expressed in Escherichia coli. The gene was expressed as apoaequorin and, by using luciferin isolated from Renilla reniformis, its activity was found essentially identical to native aequorin. The aequorin gene was subcloned into a mammalian expression vector to produce a fusion protein directing secretion of apoaequorin; the aequorin gene was fused to the 3' terminus of an immunoglobulin heavy-chain gene that directed expression of an anti-4-hydroxy-3-nitrophenacetyl antibody. The gene fusion contained the variable region, the constant region domain 1, and part of domain 2 for the IgG2b mouse immunoglobulin, followed by the aequorin gene. Transfection of the chimeric gene into a cell line expressing the complementary Al light chain, J558L, allowed recovery of a chimeric antibody with binding specificity for the 4-hydroxy-3-nitrophenacetyl group and the related 4-hydroxy-3-iodo-5-nitrophenacetyl hapten. The Ca\(^{2+}\)-dependent bioluminescent activity of aequorin was also recovered.

A variety of reporter molecules, such as radioisotopes, fluorescent and chemiluminescent molecules, and enzymes chemically linked to antibodies, have been used to quantify antigen-antibody interactions (1). However, these chemical modifications can often lead to batch-to-batch variation, loss of binding activity, and, for the commonly used radioisotopes, shelf life. In contrast, genetically engineered chimeric antibodies in which all or part of the antibody constant region is replaced by an enzyme (2) or other protein capable of generating an appropriately amplified signal offer an alternative to chemical modification that eliminates the above disadvantages without incurring any loss of sensitivity. In yet another variation single-chain antibodies have been produced by two research groups in which the light-chain and heavy-chain variable regions are linked via a short hydrophilic polypeptide and subsequently expressed in both Escherichia coli (3) and Bacillus subtilis (4) systems. Secretion of functional antibodies either as intact antibody or fragments (Fab or Fv) has also been achieved in E. coli (5, 6) and yeast (7) expression systems. The cloning and expression in E. coli of a single-chain antibody fused to a protein toxin molecule protein has also been reported (8).

Enzyme-generated luminescence, as exemplified by the ATP-dependent firefly luciferase system (9), is a particularly attractive system for application in immunodiagnostics due to its ease of detection and high sensitivity (10). However, this system would be greatly improved if the binding protein (the antibody) and the enzyme were part of the same molecule. In this case, purification of the enzyme–antibody conjugate to homogeneity could be greatly facilitated by using immunoaffinity chromatography.

We selected the well-characterized luminescent protein aequorin from the circumoral ring of the hydromedusa Aequorea victoria. Aequorin emits light ($\lambda_{max} = 507$ nm) on oxidation of bound luciferin, an imidazopyrazine (10), in the presence of Ca\(^{2+}\). This oxidation and the resulting photon flux is generated rapidly enough to discriminate 10-12 mol of photoprotein over background (10). Because the luminescent reaction is Ca\(^{2+}\) dependent and can be detected with high sensitivity, aequorin has been used as a biological Ca\(^{2+}\) indicator (11). This generation of measurable luminescence at very low aequorin concentrations indicates that aequorin may be of value in immunoassay systems, provided a reliable and homogeneous source of the protein can be obtained and connected with an appropriate antigen-binding protein.

The recent cloning and sequencing of aequorin cDNAs has revealed that aequorin is composed of 196 amino acids and contains three EF-hand structures characteristic of Ca\(^{2+}\)-binding domains. The amino acid sequences of the Ca\(^{2+}\)-binding sites of bovine calmodulin, parvalbumin, and tropomin C show strong sequence similarity to the proposed sites in aequorin (12).

Recent techniques in the stable introduction of immunoglobulin genes into myeloma cells coupled with detailed structural information has permitted use of in vitro mutagenesis methods to generate recombinant antibodies possessing special properties (13, 14). The generation of chimeric antibodies, in which the gene encoding the antigen-binding portion of the immunoglobulin is fused to the gene for another protein (for example, Staphylococcus aureus nuclease, mouse oncogene c-myc, and Klenow fragment of E. coli DNA polymerase I; refs. 2 and 15) and then expressed as a secretion protein, has already been shown with these techniques. The generality of directing secretion of an antibody-binding activity fused to other sequences and concomitantly maintaining bifunctional activities remains to be established.

To evaluate the contribution of these different factors to a luminescent immunoassay reagent we have introduced the aequorin gene from the hydromedusa A. victoria into the coding region of an antibody gene, as described by Neuberger et al. (2). When transfected into cell line J558L this fused gene directs the expression of an antibody specific for the 4-hydroxy-3-nitrophenacetyl group (NP) with the bioluminescent activity of aequorin. This chimeric antibody can also be regenerated and activated to produce light while immobilized to a solid-phase antigen, demonstrating its usefulness in an immunoassay format.

Abbreviations: NP, 4-hydroxy-3-nitrophenacetyl; NIP, 4-hydroxy-3-iodo-5-nitrophenacetyl; BSA, bovine serum albumin; PBS, phosphate-buffered saline; Eco-gpt, Escherichia coli xanthine-guanine phosphoribosyltransferase.

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MATERIALS AND METHODS

Materials. Restriction enzymes, reagents for generation of cDNA, and all radioisotopes and cloning kits were purchased from Amersham. The oligo(dT)-cellulose and oligonucleotide linkers were purchased from Bethesda Research Laboratories and Biolabs (Beverly, MA). The reagents for the A phage cloning were purchased from Stratagene. Coelenterazine, was purchased from London Diagnostics (Eden Prairie, MN).

cDNA Cloning. The jellyfishes A. victoria were collected at Friday Harbor Laboratories, University of Washington. The outer margin of the jellyfish was excised, as described by Johnson and Shimomura (16). Twenty outer margins were extracted as described (17). To the aqueous phase from the extraction, 3 vol of ethanol were added and transported back to the laboratory on dry ice. Isolated crude RNA was further purified, as described (17, 18); poly(A)* RNA was precipitated and stored at -70°C.

Double-stranded cDNA was prepared essentially as described by Gubler and Hoffman (19). EcoRI linkers were added, as described (20). The linker-d cDNA was size-fractionated on an agarose gel, and cDNA of 400–2000 base pairs was isolated by electroelution onto DEAE membranes (NA45 paper; Schleicher & Schuell). The purified linker-d cDNA was then ligated to Agt10 dephosphorylated arms (Stratagene) and packaged in vitro into λ phage by using Gigapack (Stratagene), according to standard procedures (21).

The packaged plasmids were plated on C600H11 cells, and plaques were replicated onto nitrocellulose filters for screening by hybridization with standard methods (22). Oligonucleotide probes based on the aequorin gene sequence were synthesized by an Applied Biosystems machine (12) (5'-ACAAAGGACGACCTTTACCATCTCATGAGATTGTGCTGTTACCA, and 5'-GGTGT- TACCAAAATCTCTAGTGGTTGTCCTGATCATCTCATCAACATCGAGT GTGTCACCTTC). These oligonucleotides were labeled by nick-translation with biotin 11-dUTP (Bethesda Research Laboratories); standard methods were used to generate the probe (17). After hybridization to the probe, the filters were developed with the avidin–alkaline phosphatase system (Bethesda Research Laboratories). Positive clones were selected by autoradiography, and then phage purified with solid-phase anti-λ phage antibodies (Lambda Sorb; Promega Biotec). The phage DNA was then digested with EcoRI, and insertions were subcloned into pUC18 (23) at the EcoRI site. The methods used in construction of the expression vectors were as described (17). The isolated gene was sequenced by the method of Chen and Seeburg (24).

Construction of Antibody–Aequorin Chimera. Construction of the fusion required modification of the EcoRI aequorin gene fragment to clone the gene into the Xho I site of vector 186. Vector 186 contains the genomic heavy-chain variable region specific for NP, a DNA region of the heavy-chain constant region, the hinge of genomic IgG2b, and the gene for E. coli xanthine-guanine phosphoribosyltransferase (Eco-gpt) under control of the simian virus 40 early promoter (14) (see Fig. 1 for overall construction scheme). The aequorin gene was isolated as a HindIII–EcoRI 630-base-pair fragment in which the 5' end of the gene was removed. This fragment was then ligated to a gene cassette derived from two sequences: 5'-TTAGGAGACAAGACAGTCGA and 5'-CTTGGTGGTGTATGATGTCAGTCCA.

Ligation of this gene cassette tailors the aequorin gene as a Xho I–EcoRI fragment. This tailored aequorin gene was then ligated into vector pXSM13- cut with Xho I and EcoRI, generating pXR3. The vector pXSM13- was derived from pBSM13- (Stratagene) by ligation of a Xho I linker at the HindIII site. The plasmid pXR3 was then modified by ligation of Xho I linkers to the EcoRI-digested and DNA polymerase-treated EcoRI site at the 3' end of the aequorin gene, generating the plasmid pXR3i.

The aequorin gene was isolated from plasmid pXR3i as a Xho I fragment and ligated into vector 186 to generate the plasmid p205GTI for expression of the antibody–aequorin fusion upon transfection of the J558L cell line. The sequence at the junction site between the immunoglobulin gene and aequorin is shown in Fig. 1 Lower.

Transfection of DNA into Myeloma Cells. J558L cells at 106 cells per ml of growth medium (5% fetal calf serum in Iscove's modified Dulbecco's medium/penicillin/streptomycin) were pelleted and washed with ice-cold phosphate-buffered saline (PBS) without MgCl2 or CaCl2. After being repelleted, cells were suspended at 107 cells per ml of PBS. The plasmid p205GTI, linearized by Pvu I digestion, was added to the cell suspension at 10 μg/ml. Eight-tenths milliliter of this mixture was added to an electroporation cuvette and kept on ice for 10 min.

The cells were then subjected to a high-voltage pulse of 2.0 kV/cm at 25 μF (t = 0.7 ms; Bio-Rad gene pulser) and immediately placed on ice for 10 min. Cells were then diluted into culture medium in 96-well microtiter plates. After 48 hr, cells were selected by adding growth medium containing...
mycophenolic acid at 5 μg/ml (GIBCO), xanthine at 200 μg/ml, and hypoxanthine at 15 μg/ml. ELISA assays were performed to screen for antibody production to the 4-hydroxy-3-iodo-5-nitrophenacetyl (NIP) hapten with NIP-bovine serum albumin (BSA), and positive clones were then tested for the photoprotein activity, as described.

Purification of Anti-NIP-Aequorin. NIP-agarose was made by coupling the N-hydroxysuccinimide ester of NIP-aminoacapric acid (25) to diaminopropylamine-agarose at 0.55 mg of NIP-aminoacaprole per ml of agarose. For purification, the transfected cells producing the antibody-photoprotein fusion were cultured in serum-free medium (HL-1; Ventrex Laboratories, Portland, ME) or, if cultured, in 5% fetal calf serum; the medium was adjusted to pH 5 with 1 M sodium acetate, pH 5, incubated on ice for 30 min, and centrifuged for 10 min at 7000 rpm in a Sorvall HS-4 rotor. Culture supernatant was loaded directly onto the column in PBS (1.5 mM KH2PO4/8.1 mM Na2HPO4·7 H2O/137 mM NaCl/2.7 mM KCl) and eluted with 1 mM NIP-aminoacapric acid (25) in PBS. Fractions were collected and subjected to analysis by Nanodot/PAGE, as described (26), by using 12.5% reducing and 7% nonreducing gels.

Light-producing activity of the fractions was measured on samples rererminated with the coelenterazine in 1% 2-mercaptoethanol/coelenterazine, at 0.34 ng/ml/30 mM Tris-HCl, pH 7.6/10 mM EDTA for 3 hr at 0°C.

Samples of the regenerated photoprotein were placed in the Berthold luminometer, and 30 mM CaCl2/30 mM Tris-HCl, pH 7.6, was injected, recording the resultant peak light intensities. This material was used to further characterize activities of the fusion protein.

To obtain the best light-output levels a titration of coelenterazine was done as follows: anti-NP-aequorin protein at 47 ng/ml was regenerated in 0.3 M Hepes, pH 7.5/10 mM EDTA/0.1% BSA/1% 2-mercaptoethanol with various amounts of coelenterazine for 16 hr at 4°C. These regenerated samples were assayed for light activity as above.

Photoprotein Assay. The coelenterates Renilla reniformis were collected at the Baruch Institute of Marine Biology, University of South Carolina. The luciferin was extracted using the method of Horii et al. (27). This crude luciferin preparation was redissolved in 1 M HCl in methanol for use in the aequorin photoprotein assays.

The assay of bacterial extracts was as described by Inouye et al. (28) but with the addition of 4 μl of luciferin extract per ml of extract. The culture supernatants from the transfected myeloma cell lines were diluted into assay buffer [30 mM Tris-HCl, pH 7.6/10 mM EDTA/1% (vol/vol) 2-mercaptoethanol/luciferin extract at 4 μl/ml] and kept on ice for 2 hr. This assay procedure was modified later by incorporating synthesized coelenterazine (London Diagnostics), as described below. The regenerated photoproteins from bacteria or myelomas were assayed in a Berthold luminometer, Biolumat LB 950C (Berthold Instruments, Pittsburgh) by use of 100-μl sample volumes and injection of 100 μl of 30 mM CaCl2/30 mM Tris-HCl, pH 7.6; the peak intensity was taken from the luminometer in its peak/auto-injection mode.

Activation of Anti-NIP-Aequorin on Solid-Phase Antibiotics. Dilutions of BSA labeled with NIP [NIP-BSA; prepared by coupling NIP-aminoacapric acid to BSA at a molar ratio of 4:1, according to a published method (25)] were made in 10-fold increments by using 0.05 M carbonate, pH 9.4, and used to coat tubes overnight at 4°C or at 37°C for 2 hr. Tubes were washed, blocked, and incubated for 2 hr at 37°C with 200 μl of culture supernatant containing anti-NP-aequorin. Tubes were washed; 100 μl of regeneration buffer (as above) was then added and incubated at 4°C for 24 hr. Light activity was assayed as described above.

RESULTS

Approximately 0.9 mg of RNA and 13 μg of poly(A)+ RNA per gram (wt) of outer margins were isolated from A. victoria. The yield of recombinant phage plated on C600Hf- cells, which only allows growth of recombinant phage, showed a recovery of 5 × 10^6 colonies per μg of poly(A)+ RNA starting material. From the 8400 clones screened, 20 positive clones were identified with this method, indicating an abundance of 0.2% in the enriched gene library. These data agree well with the reported 0.1% aequorin clones (29). The difference, if significant, is probably due to the size fractionation used in our study. We selected nine of the positive clones for further analysis by EcoRI digests. Three of these clones had inserts of the expected ~600-base-pair size and were subcloned into pUC18. (Fig. 1 shows construction of the expression vector.) The HindIII–EcoRI fragment containing the aequorin gene was isolated from the subclone with the largest EcoRI insert. This gene fragment was recloned into a Xho I–EcoRI-digested pXSM13, as described. The resultant clone pXR3 allows expression of a lacZ–aequorin gene in E. coli via the lac promoter present in pBSM13; thus, the correct clone was potentially identifiable by its activity. To determine whether photoprotein activity was recoverable in lysates from E. coli cultures on induction of lac promoter, we tested lysates and found photoprotein activity at low levels (data not shown). The clone pXR3 was further modified, as described. The resultant clone pXR3i was used as the source of the Xho I fragment for introduction into vector 186 (2) at the Xho I site, generating plasmid p205GTi and allowing for the potential expression of the aequorin gene fused to antibody sequences.

Transfections by electroporation yielded ~100 anti-NIP-immunoglobulin-positive clones per μg of plasmid DNA, with 1 cell in 10^8 cells being transfected. Four of the culture supernatants from the anti-NIP-immunoglobulin-positive cell lines were assayed for photoprotein activity and found to contain similar levels of activity (range of 2-fold). This narrow range of expression levels was found for previous transfections when using these methods (data not shown).

Characterization of the Chimeric Protein. To further characterize the product of the antibody–aequorin gene fusion on plasmid p205GTi, affinity purification of the NIP-binding activity was done as described above.

The profile of an ELISA, measuring antibody-binding activity and the profile of the light activity (data not shown), demonstrates copurification of the antibody and photoprotein activity.

Analysis of the purified material by gel electrophoresis demonstrated protein bands with Mf values of 74,000–76,000 on the nonreducing gel (Fig. 2 Upper, lane 2) and, upon reduction, proteins of 26,000 (the λ light chain) and a doublet at Mf 50,000–52,000 (Fig. 2 Upper, lane 1). These values agree with the molecular weight calculated from the aequorin sequence used plus the variable region of the heavy chain–NP Fab fragment. Furthermore, the Mf derived from the nonreducing gel indicates that the chimeric antibody exists as a Fab′ rather than a (Fab′)2 fragment (see Fig. 2 Lower). The expected molecular structure is illustrated in Fig. 2 Lower for comparison.

The fusion product was further characterized for its photoprotein activity by titration of the substrate coelenterazine (Fig. 3). The data are similar to others, as far as comparison can be made lacking detailed data for apoaequorin (28).

To investigate the usefulness of the protein in an immunosassay format, we determined the ability of the chimeric protein, bound to antigen-coated tubes, to regenerate photoprotein activity. Fig. 4 shows that when chimeric protein bound to solid-phase antigen, coelenterazine addition caused regeneration of normal aequorin activity.
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**Fig. 2.** (Upper) Molecular weight analysis of the affinity-purified antibody–aequorin protein. Samples were subjected to NaDodSO₄/PAGE analysis by using 12.5% reducing (lane 1) and 7% nonreducing (lane 2) gels. The $M_r$ values determined from marker-protein migration are indicated as $M_r \times 10^{-3}$. (Lower) Predicted primary structure of the anti-NP antibody, including the expected $M_r$ values of the components. Disulfide links (−SS−) between the heavy (H) and light (L) chain are indicated. V, variable region; C$_{\gamma 2\lambda}$, constant region of IgG2b; h$_{2b}$, hinge region of IgG2b.

**DISCUSSION**

A number of aequorin gene sequences have been described (28, 29), and comparison of the sequence reported here shows that our sequence differs from that described by Inouye et al. (28) at only seven positions, all of which are confirmed silent nucleotide changes. This may indicate gene polymorphism or a multigene family. In this regard, evidence exists from others (29, 30) of multiple aequorin genes and proteins, the sequences and possibly properties of which differ from those of Inouye et al. (28) and ourselves.

**Fig. 3.** Effect of coelenterazine concentration on regeneration of antibody–aequorin light activity. Light output was measured in counts/s on Ca²⁺ addition to samples of regenerated antibody–aequorin, as described. Light output was integrated over a 10-s period.

**Fig. 4.** Binding of the antibody–aequorin chimera to solid-phase antigen and recovery of light activity on adding coelenterazine in the indicated amounts. Luminometer tubes were coated with NIP-BSA. Samples of antibody–aequorin were incubated with antigen-coated tubes before washing and coelenterazine addition. Light activity was measured on Ca²⁺ addition and integrated over a 10-s period.

The potential utility of aequorin in assay systems had not been realized due to the sensitivity of the aequorin–coelenterazine complex to various chemical methods of labeling and conjugation, which prevented its effective use as a reporter molecule (31). The availability of recombinant aequorin, the stability of which is enhanced relative to holoenzyme and the activity of which can be fully recovered on addition of coelenterazine, may allow development of more flexible assay formats. In particular, the possibility exists of formatting immunoassays where Ca²⁺ ions are present in the initial binding stages but are subsequently removed by washing before activation with coelenterazine.

Our aim in constructing this chimera was to evaluate the potential use of aequorin in immunoassays. The gene fusion generated contained the heavy-chain variable region and IgG2b of the constant region of domain 1 segments of anti-NP antibody [as described by Neuberger (15); see Fig. 2 Lower] fused to the 5' end of the aequorin gene. NaDodSO₄/PAGE analysis of the purified antibody–aequorin fusion protein showed species with $M_r$ values consistent with the predicted product of the gene fusion and its association with the light chain expressed by the J538L myeloma. The finding that most unprocessed material was present in a Fab'-like form with a $M_r$ of 74,000–76,000 (Fig. 2 Upper, lane 2) was of interest but not unexpected, as the S. aureus nuclease fusion generated by Neuberger et al. (2) had indicated some interference with dimerization to a (Fab')₂-like molecule in such gene fusions. The addition of the light chain is not affected to a significant extent, as shown by the presence of the light chain in the purified product and the copurification of NIP-binding activity and aequorin activity.

In analyzing affinity-purified antibody fusion on reducing gels (Fig. 2 Upper, lane 1) a doublet of bands was resolved at the position for the gene-fusion product. This phenomenon had been seen for fusion of the S. aureus nuclease fusion generated by Neuberger et al. (2).

Overall, the biochemical analyses provided good evidence that the bifunctional fusion protein was the major species purified. In functional assays, the expressed fusion protein in the supernatant from transfected cells could use either natural or synthetic coelenterazine in generating luminescence and recovery of photoprotein activity. Furthermore, affinity purification of the culture supernatants on a solid-phase antigen copurified photoprotein activity (data not shown). These results provide firm evidence that the major species purified was a bifunctional fusion protein.
The photoprotein activities of the aequorin chimera are similar to those reported for aequorin (28, 29, 32), though stability studies indicate that the construct described here exhibits greater stability than other preparations in prolonged storage as the holoprotein (data not shown). This result was unexpected because published results (31) have suggested that the holoprotein is relatively unstable.

In general, the chimeric protein does not show any significant differences in its photoprotein activity compared with the data available for the native- and bacterial-expressed proteins (28, 29). Data from the kinetics of light output, where peak durations are 2–3 s (data not shown), indicate that light-production characteristics from the fusion protein are near those of native aequorin (33). This fact suggests that other modifications of the N terminus of aequorin are possible, given that addition of a 77 kDa immunoglobulin Fab has no affect on its activity.

Toward the goal of site-directed labeling of aequorin, we have evaluated the potential of the Fab portion of the fusion protein to act as a target for direct (e.g., biotin) labeling procedures that allow streptavidin capture (unpublished data). The ability of the antibody–aequorin protein to bind to immobilized antigen and subsequently undergo aequorin reactivation has demonstrated its potential in immunoassay development (Fig. 4). The general utility of this procedure will be further apparent when apoaequorin fusion proteins are produced that contain high-affinity antibody-binding sites for hormones and polypeptides (e.g., glycoprotein hormones, such as thyroid-stimulating hormone and viral antigens) of low concentrations presently difficult to measure.

In conclusion, this system for expression of aequorin is potentially valuable for several types of study. Because the fusion protein can be readily purified and relative specific activities can be accurately determined, the system offers an ideal method for structure–activity studies of the aequorin gene family by, for example, site-directed mutagenesis. The importance of a method for achieving high purity cannot be overstated because the photosensitivity of aequorin is greatly affected by Ca2+ and sulfur-containing compounds, normally present in crude preparations (34). In addition, the preliminary immunoassay results presented here suggest that chimeric antibody–aequorin proteins will find an important application in high-sensitivity immunoassays.

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