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GALACTOSE-DEPENDENT EXPRESSION OF THE RECOMBINANT CA^{2+}-BINDING PHOTOPROTEIN AEQUORIN IN YEAST

Junko Nakajima-Shimada, Hidetoshi Iida,
Frederick I. Tsuji*, and Yasuhiro Anraku

Division of Cell Proliferation, National Institute for Basic Biology, Nishigonaka, Myodaijicho, Okazaki 444, Japan

*Osaka Bioscience Institute, Furuedai, Suita Osaka 565, Japan and Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093

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Aequorin is a Ca^{2+}-binding protein that emits light upon reacting with Ca^{2+} and has been used as a probe for monitoring changes in the intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$. The protein consists of three components: apoaequorin (apoprotein), molecular oxygen and a chromophore. The present study was designed to conditionally express the apoaequorin cDNA of the jellyfish Aequorea victoria under the control of the GAL1 promoter in the yeast Saccharomyces cerevisiae and to investigate whether apoaequorin can be accumulated in high enough concentration in the cells to detect a Ca^{2+} signal in vitro. The results showed that the cells accumulated sufficient amounts of recombinant apoaequorin when incubated in the galactose-based medium and that the protein was active and not toxic to the cells, suggesting that the recombinant apoaequorin may be applicable to monitoring changes in $[Ca^{2+}]_i$ in intact yeast cells.

The bioluminescent jellyfish Aequorea victoria possesses a monomeric Ca^{2+}-binding protein, aequorin (Mr=21,400), which emits light upon reacting with Ca^{2+} (1-3). The protein is composed of apoaequorin (apoprotein), molecular oxygen and a tightly bound chromophore, coelenterazine (4,5). The binding of Ca^{2+} to aequorin results in an intramolecular reaction in which

ABBREVIATIONS

EDTA, ethylenediaminetetraacetic acid; 2-ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; IPTG, isopropyl β-D-thiogalactopyranoside; $[Ca^{2+}]_i$, the intracellular free Ca^{2+} concentration.

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coelenterazine is oxidized to coelenteramide, yielding light (λ_{max}=470 nm), CO_2 and a blue fluorescent protein (5,6). Aequorin is regenerated from apoaequorin by incubation with coelenterazine, molecular oxygen, 2-mercaptoethanol (2-ME), and ethylenediaminetetraacetic acid (EDTA) (7). The primary structure of apoaequorin has been deduced from the nucleotide sequence of the cDNA (8) and determined by amino acid sequence analysis of the protein (9).

Although aequorin has been used as a probe for measuring $[Ca^{2+}]_i$ (10,11), the use of this protein has been limited by the difficulty of introducing sufficient aequorin into small cells to detect a measurable signal. Many methods for aequorin loading have been used in the past, such as microinjection (11), hypoosmotic shock (12), scrape loading (13), and gravity loading (14). These methods require expensive, purified aequorin and may injure the cells. Expression of the cDNA for apoaequorin in cells may overcome these problems.

Essential roles for Ca^{2+} in the cell cycle and the mating process of the yeast Saccharomyces cerevisiae have been discovered (15,16). It would therefore be desirable to establish a system for monitoring rapid changes in $[Ca^{2+}]_i$ in this organism. The present study was designed to express the apoaequorin cDNA under the control of the GAL1 promoter so that the cDNA is expressed only when galactose is used as a carbon source, and to ask whether apoaequorin can be accumulated in sufficient concentrations in yeast cells so as to be able to detect a measurable signal in vitro and whether the protein is toxic to yeast cells when expressed at high levels.

MATERIALS AND METHODS

Media and Chemicals: Rich medium (YPD), synthetic glucose-based medium (SD), and synthetic galactose-based medium (SSG) were prepared as described by Iida et al. (16). These synthetic media

were supplemented with appropriate auxotrophic requirements. Aequorin used for making calibration curves was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Calcium ionophore A23187 (free acid) was from Sigma. Coelenterazine [2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-3,7-dihydroimidazo[1,2-a]-pyrazin-3-one] was prepared by chemical synthesis (17). Other chemicals were standard commercial products of the highest grade.

Yeast and Escherichia coli Strains: Yeast strain used in this study was SP1 (MATa his3 leu2 ura3 trp1 ade8 can1) (18). Yeast transformations were performed by the lithium acetate method (19). The E. coli strains HB101, JM83 and dam 3704 (20) were used for propagation and isolation of plasmids. JM83 was also used for expression of apoaequorin.

Plasmids: The plasmid piQ5 containing the apoaequorin cDNA was previously described (21). Synthetic deoxyoligonucleotides , 5'-CCGCGGGATGGTCA-3' and 5'-AGCTTGACCATCCCGCGG-3' were synthesized by Applied Biosystems Japan, Inc., annealed to each other and used as a linker. The plasmid piQ5L was constructed by inserting this linker to the ClaI/HindIII fragment of piQ5. The cloning vector YCp103, kindly provided by S. Harashima, is based on a YCp50 vector and contains the promoter of the GAL1 gene (22) which is located upstream from a multilinker site. The selection marker used for yeast cells was URA3. YCpAQ1 was constructed by joining the EcoRI/SacII 0.6 kb fragment of the apoaequorin cDNA in piQ5L with the BamHI/SacII 8.6 kb fragment of YCp103. The nucleotide sequence of the junction region in YCpAQ1 plasmid was determined with the T7 Sequencing™ Kit (Pharmacia LKB Biotechnology).

Assay for aequorin activity in vitro: Yeast cells carrying YCpAQ1 or YCp103 and E. coli (strain JM83) cells carrying piQ5 were harvested by centrifugation. Cell disruption was achieved with glass beads for yeast cells and with sonication for E. coli cells as described previously (21,23). After addition of 100 μl of 30 mM Tris-HCl, pH 7.60/ 10 mM EDTA/ 1 mM PMSF, the disrupted cells were vortexed and centrifuged for 10 min at 10,000 × g at 4℃. The supernatant (hereafter called the "extract") was used for the assays as described by Inouye et al. (21). After appropriate dilutions, the diluted extracts (100 μl) received 1 μl of 14.2 M 2-ME and 0.6 μl of 1 mg/ml coelenterazine dissolved in 99.5 % methanol. After incubation on an ice-bath for 2 h, 2.5 μl of the above mixture was injected into 500 μl of 30 mM $CaCl_2$ / 10 mM Tris-HCl, pH 7.60. The initial maximal light intensity was measured at 25℃ by a photomultiplier photometer (Model CAF-100, Japan Spectroscopic Co., Tokyo), and expressed as an arbitrary unit.

Western blot analysis: Yeast cells carrying YCpAQ1 or YCp103 were harvested by centrifugation, disrupted with glass beads (23), and subjected to electrophoresis using 12.5% polyacrylamide gels containing SDS (24). Proteins were electrophoretically transferred to a membrane, Immobilon™ (PVDF; Polyvinylidene difluoride, Millipore Corp., Bedford, MA). The membrane was incubated with rabbit antiserum against aequorin and then with peroxidase-labeled anti-rabbit IgG.

RESULTS AND DISCUSSION

As an initial step in our study to establish an experimental system for monitoring rapid changes in $[Ca^{2+}]_i$ in yeast cells, we

investigated whether or not apoaequorin is toxic to the cells. The plasmid YCpAQ1 in which the apoaequorin cDNA was fused downstream from the GAL1 promoter was constructed by ligating the EcoRI/SacII 0.6 kb fragment containing the cDNA with the BamHI/SacII fragment of the expression vector YCp103, as illustrated in Fig. 1. In YCpAQ1 the cDNA is expressed only when galactose is used as a carbon source and repressed when glucose is used. Cells carrying YCpAQ1 or YCp103 growing exponentially in SD medium was shifted to SSG medium, and growth properties and accumulation of the recombinant apoaequorin were examined (Fig. 2). Fig. 2A shows that doubling time and saturation density were essentially the same for cells carrying YCpAQ1 and those carrying YCp103 in SSG medium. Cell morphology was also the same for the two (data not shown). Fig. 2B shows that luminescence intensity in extracts prepared from the cells carrying YCpAQ1 or YCp103.

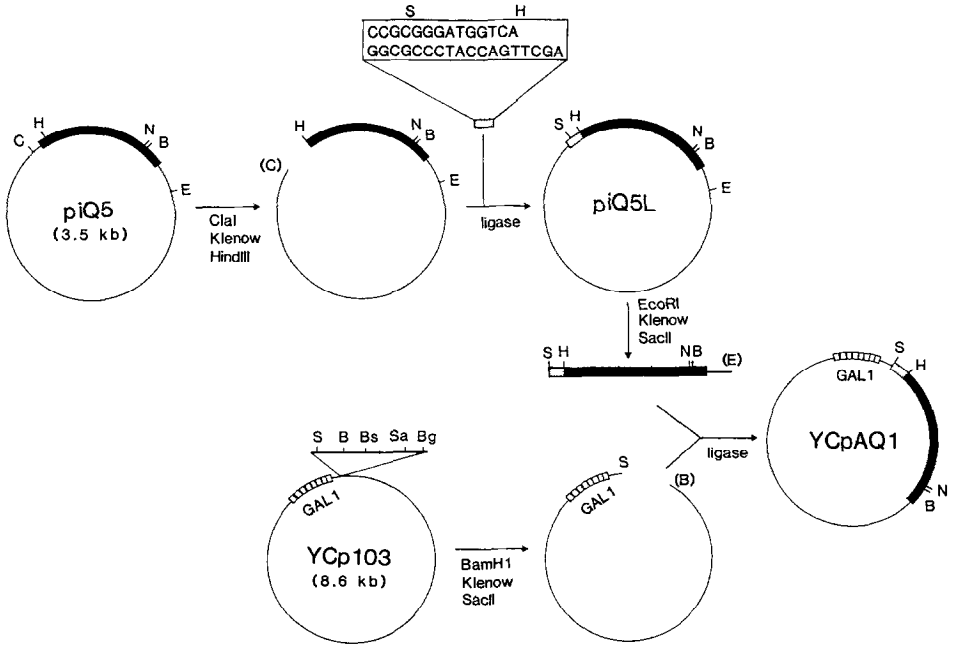

Fig. 1. Scheme for construction of the recombinant apoaequorin expression plasmid YCpAQ1. The area marked with stripe represents the GAL1 promoter, the stippled region the synthetic deoxyoligonucleotide linker, and the closed portion the apoaequorin cDNA. C, ClaI; H, HindIII; B, BamHI; E, EcoRI; S, SacII; N, NcoI; Bs, BstI; Sa, SalI; Bg, BglII.

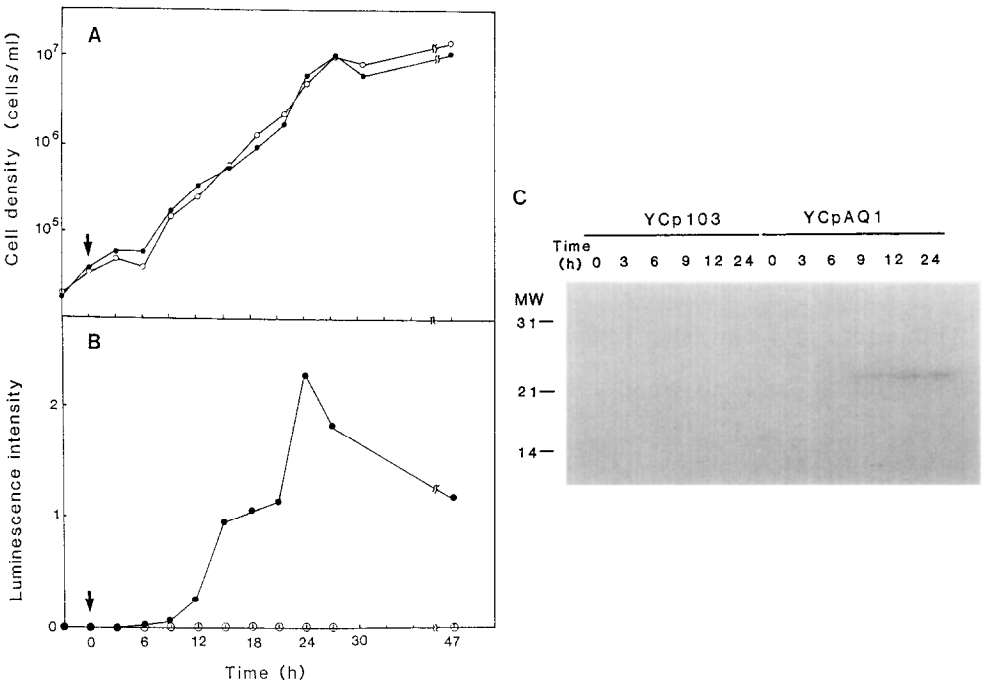

Fig. 2. Growth curve of S. cerevisiae cells carrying YCp103 or YCpAQ1 and expression of apoaequorin. (A) Growth curve of SP1 cells carrying YCp103 (○) or YCpAQ1 (●). At time 0 (indicated by an arrow), cells were shifted to SSG medium from SD medium at 30℃. (B) Expression of apoaequorin. At the indicated times, cells were harvested, and luminescence intensity of extracts (5 μl each) were measured in vitro, as described under Materials and Methods. Luminescence intensity was normalized with the content of protein contained in each extract. ○, YCp103; ●, YCpAQ1. (C) Western blot analysis. Extracts were subjected to SDS-PAGE (12.5% gel) and then to immunoblot analysis using rabbit antiserum against aequorin.

Extracts were prepared at the times indicated in Fig. 2B after the shift to SSG medium and subjected to assay for aequorin activity in vitro. The results showed that luminescence intensity in the extracts prepared from the cells carrying YCpAQ1 was detectable 6 h after the shift and increased rapidly thereafter until the cells entered the stationary phase. Quantification using a calibration curve relating the amount of aequorin versus luminescence intensity indicated that the intracellular content of the recombinant apoaequorin was 0.38 ± 0.15 μM at 15 h after the shift. Expression of the recombinant apoaequorin was confirmed by Western blot analysis using anti-aequorin antibody (Fig. 2C). Neither luminescence activity nor a protein band was

detected in extracts prepared from control cells carrying YCp103 (Fig. 2B and 2C). These results indicated that the recombinant apoaequorin produced in yeast cells is active and not toxic.

Since the plasmid piQ5, which contains the apoaequorin cDNA downstream from the tac promoter, has been found to give excellent expression of apoaequorin in E. coli cells treated with an inducer of the tac promoter, IPTG (21), we compared the ability of YCpAQ1 to produce active apoaequorin in yeast cells with that of piQ5 in E. coli cells. Extracts were prepared from yeast cells carrying YCpAQ1 grown for 15 h in SSG medium and from E. coli cells carrying piQ5 incubated for 2 h with IPTG. Aequorin was regenerated and the assay for aequorin activity was carried out as described in Materials and Methods. The results showed that the activity in the yeast extracts was comparable to that in the E. coli extracts (Fig. 3). These results provide for

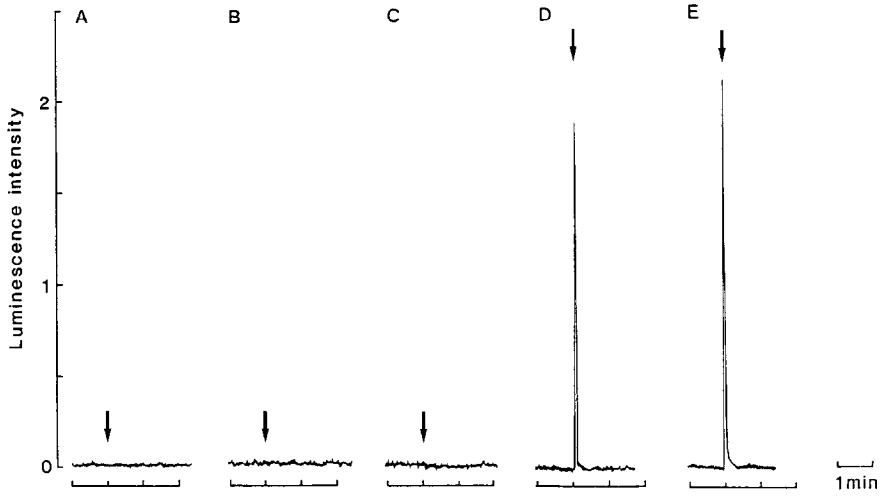

Fig. 3. Assay of aequorin regenerated in vitro from extracts prepared from S. cerevisiae and E. coli cells. Exponentially growing yeast cells carrying YCp103 or YCpAQ1 in SD medium and those that had been incubated for 15 h in SSG medium were harvested and subjected to protein extraction as described under Materials and Methods. Cells of E. coli JM83 carrying piQ5 were incubated with IPTG for 2 h at 37°C and subjected to extraction. Extracts (0.1 μg each of protein) prepared from these cells were subjected to in vitro assay as described under Materials and Methods. An aliquot (10 μl) of the assay mixture was added at the position indicated by an arrow. Extracts prepared from yeast cells carrying A, YCp103 in SD medium; B, YCpAQ1 in SD medium; C, YCp103 in SSG medium; D, YCpAQ1 in SSG medium. E, Extracts prepared from E. coli cells carrying piQ5.

the possibile use of recombinant apoaequorin as an intracellular probe for monitoring rapid changes in $[Ca^{2+}]_i$ when coelenterazine is introduced into intact yeast cells and aequorin is regenerated *in vivo*.

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