A Novel Renilla Luciferase/Epidermal Growth Factor Fusion Protein as an Optical Molecular Probe for Cancer Imaging

Andreas M. Loening Ramasamy Paulmurugan Anna M. Wu Sanjiv S. Gambhir

Crump Institute for Molecular Imaging, UCLA School of Medicine
Department of Radiology and the Bio-X Program, Stanford University

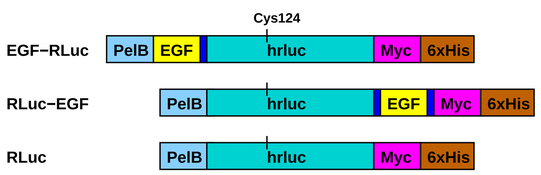
INTRODUCTION

Our lab is working on developing novel probes for imaging receptor expression using *in vivo* optical techniques. One potential target, epidermal growth factor (EGF) receptor, is up-regulated in many forms of cancer [1]. In light of this, we have pursued the construction and validation of a Renilla Luciferase (RLuc)/EGF fusion protein as a probe for the study of the EGF receptor system *in vivo*.

CONSTRUCTS

	AA's	Size (KDa)	pI
RLuc	335	38.8	5.9
EGF-RLuc	389	45.0	5.7
RLuc-EGF	390	45.1	5.7
RlucC124A	335	38.8	5.9
EGF-RLucC124A	389	45.0	5.7
RLucC124A-EGF	390	45.1	5.7

Wild Type Constructs



The above constructs were assembled via PCR cloning. CMV-hRLuc plasmid (Promega) was used as the initial template, and the DNA sequence encoding the 53 amino acids of mature human EGF was extended onto either the 5' or 3' end of the hRLuc gene, with single glycines (dark blue) used as spacers. The pelB leader sequence was then appended to the 5' end to provide a signal for protein export to the bacterial periplasm.

The final fusion genes were then cloned into the pBAD/Myc-HisA plasmid (Invitrogen) such that the Myc epitope and 6xHistidine tag were attached to the fusion construct. The pBAD plasmid is a bacterial expression vector in which the protein of interest is under the control of an inducible arabinose promoter.

C124A Constructs

In addition to the wild type constructs shown above, constructs with a Cys to Ala mutation at position 124 of RLuc were produced using a QuikChange mutagenesis kit (Stratagene). Liu and Escher [2] have previously shown that this mutation (Cys152 in their construct) improves the stability of the Renilla protein, and comparable results were seen in our hands.

PURIFICATION

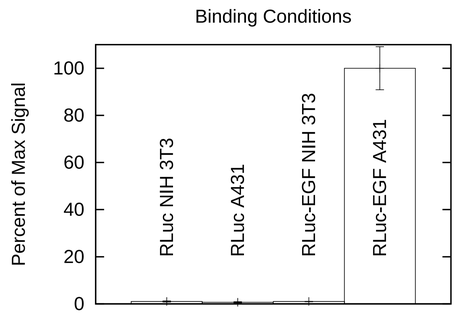
Protein production was performed in *E. coli* LMG 194 cells grown at 32° C. Cultures were allowed to come to an OD_{600} of 0.7, and arabinose was added to a final concentration of 0.2%. Two hours later, cells were harvested and the periplasm extracted by osmotic shock using the method of Neu and Heppel [3].

The periplasm was brought to 1 mM PMSF, and then purified by nickel affinity chromatography (Ni-NTA Superflow, Qiagen). The chromatography buffer consisted of 300 mM NaCl and 20 mM HEPES at pH 8, with 20 mM imidazole in the loading and washing steps and 250 mM imidazole in the elution step. Human serum albumin was added to 1% as a carrier protein, and the elution was then desalted using a PD-10 column. This partially purified protein solution was stored for later use in 20% glycerol at −80° C.

IN VITRO EVALUATION

Specificity

Specificity of the fusion protein was assessed by comparing binding of RLuc-EGF to RLuc on both A431 human epidermoid carcinoma cells (high EGF receptor expression) and NIH 3T3 murine fibroblasts (negative control). Cells were plated at $1 * 10^5$/well in 24 well plates and allowed 24 hours to attach. 8000 RLU's/well of either fusion or control protein was then applied in 500 μl/well of media without FBS. Following a 60 minute incubation, the media was aspirated, the cells washed twice with PBS, and the wells refilled with 500 μl/well PBS. 0.5 μg/well coelenterazine was added, and the plate was imaged in a Xenogen bioluminescence optical system.

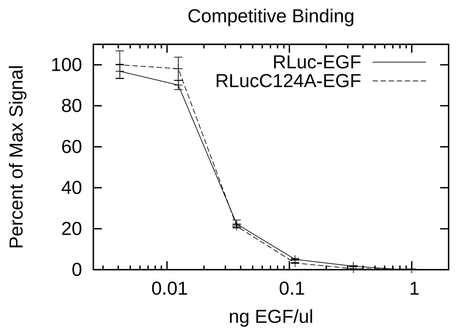


The results show the RLuc-EGF fusion protein is specific for the high EGF receptor expressing A431 cells, indicating that the EGF portion of the fusion protein is responsible for the binding to these cells. Initial results for the EGF-RLuc fusion protein gave poor binding results (not shown), and this construct has not been further pursued.

Competitive Binding

A competitive binding experiment was performed to further confirm the specificity of the RLuc-EGF fusion protein for the EGF receptor.

A431 cells were plated at $1 * 10^5$/well and allowed to attach for 24 hours. RLuc-EGF at 8000 RLU's/well was added in 500 μl/well of media without FBS along with an escalating dose of EGF (human recominant, PepropTech). Following a 40 min incubation, washing and analysis was done as described above. The experiment was later repeated with the RLucC124A-EGF fusion protein with similar results.



Serum Stability

Serum stability was measured to assess the feasibility of using these constructs *in vivo*. 25 μl of freshly prepared murine serum was added to 5 μl of purified protein and incubated at 37° C. 1 μl samples were taken at 10 minute intervals over the course of 2 hours and measured for luciferase activity by combining with 100 μl sodium phosphate buffer (pH 7) and 0.5 μg coelenterazine. The Cys124Ala mutation showed enhanced serum stability compared to wild type for both cases.

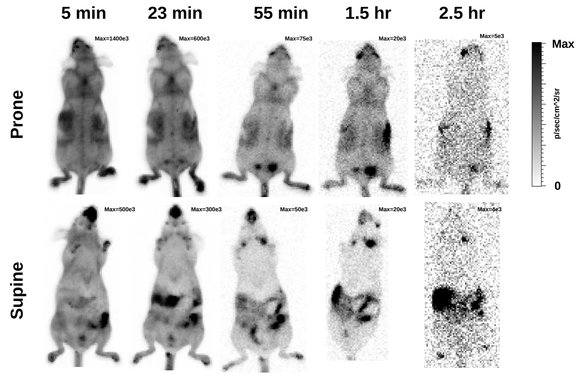
	Serum Stability ($\tau_{1/2}$)
RLuc	20 min
RLuc-EGF	10 min
RlucC124A	900 min
RLucC124A-EGF	70 min

IN VIVO EVALUATION

Distribution of RLucC124A

The distribution of RLucC124A was assessed in non-tumor being athymic (nude) mice to check the persistence of Renilla activity *in vivo*. RLucC124A protein ($\sim 250 * 10^6$ RLU's) was injected *iv* at $t = 0$, and *iv* injections of 10 μg coelenterazine immediately preceded each imaging time point.

A representative mouse is shown below. The results demonstrated that imaging of *iv* injected Cys124Ala mutated Renilla is feasible over a matter of hours.

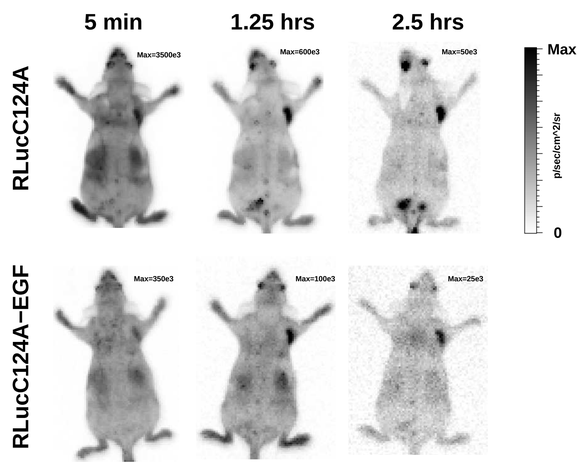


Tumor Models

Tumor models were constructed by injecting $1 * 10^6$ A431 cells subcutaneously into the right shoulder of athymic (nude) mice. The tumors were allowed to grow until palpable ($\sim$10 days) prior to use. For imaging, RLucC124A-EGF was injected *iv*, with scans performed in the prone position at 5 minutes, 1 hour, and 2.5 hours. An *iv* injection of 10 μg coelenterazine immediately preceded each of these scans. On the following day, the imaging protocol was repeated with RLucC124A instead.

The total activity of protein used was limited by the injection volume, with $\sim 100 * 10^6$ RLU's of RLucC124A-EGF and $\sim 250 * 10^6$ RLU's of RLucC124A being used per mouse.

Shown below is a representative mouse study. At the early time points examined so far, specific binding of the fusion protein to the A431 tumor cannot be distinguished from non-specific processes. The non-specific processes involved could be either protein extravasation through leaky tumor endothelium and/or tumor hypervascularization and resultant increased blood pool signal. In either case, due the quick kinetics of degradation for the fusion protein, later time points at which non-specific signal might clear cannot currently be imaged.



CONCLUSION

- A bifunctional RLuc-EGF fusion protein has been evaluated *in vitro*, with specificity demonstrated by cell binding assays and competitive binding experiments with native EGF.
- *In vivo* and *in vitro* stability of Renilla luciferase was shown to markedly improve following the incorporation of the Cys124Ala mutation.
- Following *iv* administration, mutant Renilla luciferase activity could be imaged *in vivo* for up to 2.5 hrs.
- Tumor bearing mice showed enhanced luciferase activity in the tumor following *iv* injection of the RLucC124A-EGF protein, but this enhanced activity could not be demonstrated to be specific to binding of the EGF receptor.

References

[1] Klijn JGM, et al. *Endocr Rev*, 13:3–17, 1992
[2] Liu J, et al. *Gene*, 237:153–159, 1999
[3] Neu HC, et al. *J Biol Chem*, 240:3685–3692, 1965

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