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14. ABSTRACT:
We aim to develop protein therapeutics that neutralize growth factors that activate EGF receptor family members in breast cancer. Rather than targeting receptors themselves (as do Herceptin, Iressa, etc), we propose to target the activating ligands. Our model is Argos from Drosophila, which we showed naturally inhibits EGF receptor signaling in fruit flies by inactivating the ligand. We hope to effectively 'humanize' Argos - making it bind human EGFR ligands and/or to use human protein scaffolds for this. In the past year, we crystallized a complex between the minimal functional fragment of Argos and its target (Spitz), and are about to complete structure determination – which will provide critical information for therapeutic design. We also established an experimental approach for screening libraries of Argos variants for those that bind human EGF-like ligands (our therapeutic aim). This approach employs yeast surface (rather than phage) display. We are now poised to combine our technical position and new structural information to identify Argos (and Dkk) variants that bind human EGFs and represent starting points for developing new therapeutics.

15. SUBJECT TERMS
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INTRODUCTION:

Tuberous sclerosis complex (TSC) is an autosomal dominant disease characterized by the presence of benign tumors, called hamartomas. TSC occurs due to mutations in either of two genes: hamartin or tuberin. Tuberin/hamartin complex is a negative regulator of the small GTPase, Rheb. Rheb activates mTOR, one of the master regulators of cell size. In the absence of functional tuberin or hamartin, Rheb is active, the protein synthesis machinery is turned on by mTOR, and the cell grows in size, leading to TSC. To date, there are no known specific inhibitors of Rheb. In this proposal, we set out to identify peptide inhibitors of Rheb using a yeast two-hybrid interaction trap system. Our goal was first to identify the peptides that bind Rheb in yeast. Then, we planned to confirm that peptides that bind Rheb also inhibit its GTPase activity in vitro. Finally, we aimed to determine the ability of selected peptides to inhibit Rheb function in cultured cells.

BODY:

Task 1. To identify the peptides that bind Rheb in yeast.

We first cloned Rheb from a rat embryonic brain cDNA library using specific primers and polymerase chain reaction (Figure 1). We confirmed the sequence of this clone and generated a yeast expression plasmid with Rheb fused to Gal4 DNA-binding domain. We expressed this fusion protein in yeast and using antibodies to Gal4 and Rheb showed that a full-length fusion protein was being expressed in yeast cells. We then tested whether Rheb-Gal4 fusion protein activated Gal4-dependent transcription by itself in yeast cells. We did not detect any self-activation (growth on adenine-deficient media) with this construct allowing us to move forward with the library screen. We used this Rheb-Gal4 fusion protein as a bait to screen a combinatorial peptide library.

The peptide library was expressed within the active loop of *E. coli* Trx protein, which is fused to Gal4 activation domain. If Rheb-Gal4 binds a peptide with high affinity in yeast, then the Gal4-binding domain and Gal4 activation domains come in contact, Gal-4 dependent gene, *ADE2*, is transcribed, and yeast are able to grow in the absence of adenine. By monitoring growth on a selective (no adenine) medium, we searched for yeast colonies in which there is interaction between the bait (Rheb) and the prey (peptide). Despite several rounds of screening, we did not find any colonies that could consistently grow in the absence of adenine. Results are represented in detail in Table 1.

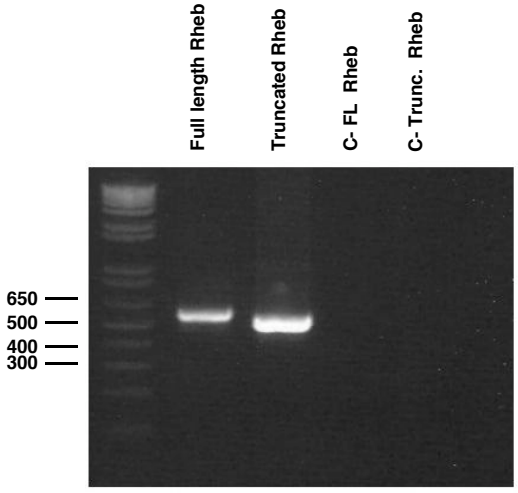

Figure 1: Full length and truncated Rheb amplified from a rat embryonic brain cDNA using specific primers and polymerase chain reaction. First lane, molecular weight markers, second lane full length Rheb (550 bp), third lane RhebΔ15 (507 bp). Last two lanes are negative controls with no template added.

TABLE 1: summary of yeast two-hybrid experiments with Rheb

Experiment #	Bait	# transformants screened	# colonies growing on no-adenine medium
1	Full length Rheb	1×10^6	None
2	Full length Rheb	2×10^6	None
3	RhebΔ15	1×10^6	None
4	RhebΔ15	2×10^6	None

To confirm that there were no technical problems with the screen (yeast strain, reagents, selection medium etc), we performed a yeast interaction screen using a different bait (mex5) in parallel with the Rheb screen. These experiments were funded by another sponsor. Using similar conditions and screening a similar number of transformants, we identified several putative interactors in this screen (Table 2 and Figure 2). These results indicated to us that there were no significant technical or quality control problems with the Rheb interaction screen.

TABLE 2: summary of yeast two-hybrid experiments with mex5 performed in parallel with Rheb

Experiment #	Bait	# transformants	# growing on selection media	Blue colonies on X-gal	In-frame sequences
5	Mex5-DHPH	1×10^6	477	72	21
6	Mex5-CT	1×10^6	239	51	10

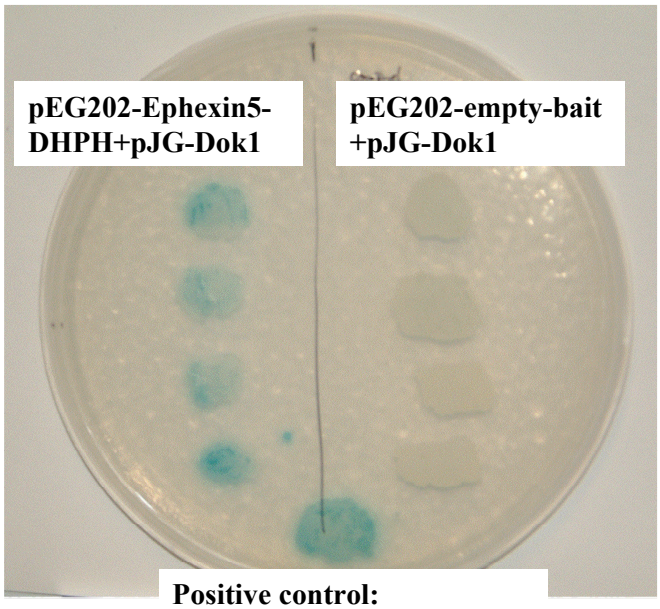

Figure 2: Yeast two-hybrid screen performed with the bait mex5-DHPH domain lead to identification of novel interactions. One of them, Dok1, is shown in this figure. Dok1 by itself does not show any self-activation (right side of the plate), but activates transcription (yeast turning blue on Xgal) when it is co-expressed with mex5 (left side of the plate). Positive control is shown at the bottom.

Having failed to identify any peptides that bind to full-length Rheb, we reasoned that this form of the molecule could have some intramolecular interactions, which inhibited its interaction with the peptide library. Rheb consists of 184 amino acid residues. The 169 N-terminal residues form the GTPase domain; the 15 C-terminal residues are hypervariable with a flexible structure and comprise a conserved motif that plays important roles in the farnesylation of Rheb and thus its membrane-association (Yu et al., 2004; Yu et al., 2005). We reasoned that if we had a truncated form of Rheb, which does not contain this flexible C-terminal, binding sites on Rheb could be more accessible to the peptide library. Using PCR, we generated RhebΔ15-Gal4, verified the sequence of this construct and expressed this fusion protein in yeast (Figure 1). We were able to confirm that RhebΔ15-Gal4 is expressed in yeast using western blotting. We then tested whether RhebΔ15-Gal4 fusion protein self-activated in yeast

cells, and it did not. We then used this RhebD15-Gal4 fusion protein as a bait to screen the peptide library again using ability to grow on adenine as the selection criteria. We did not identify any peptides that could bind RhebΔ15-Gal4 fusion protein in this screen (Table 1).

Task 2. To confirm that peptides that bind Rheb also inhibit its GTPase activity in vitro
Task 3. To determine the ability of selected peptides to inhibit Rheb function in cultured cells
Since Tasks 2 and 3 were dependent on the successful identification of peptides in Task 1, we were unable to proceed to these Tasks.

KEY RESEARCH ACCOMPLISHMENTS: none

REPORTABLE OUTCOMES: none

CONCLUSIONS: Unlike many other GTPases, Rheb appears to have low intrinsic GTPase activity and a high basal GTP level, leading to the suggestion that the wild-type Rheb acts essentially as a constitutively active protein. Thus, in the absence of Tsc2, Rheb is likely to be extremely active in cells. Therefore, identifying specific inhibitors of Rheb is an important goal in search of pharmacological treatments of Tuberous Sclerosis Complex. Despite work by many groups, this goal has not been achieved to date. Our approach of using a yeast two-hybrid screen with a combinatorial peptide library similarly failed over the course of this grant's funding period. There are alternative strategies to search for inhibitors of Rheb. New data published during the funding period of this grant provides interesting and somewhat unexpected information about the structure and function of Rheb (Urano et al., 2005; Yu et al., 2005). Furthermore, a new regulator for Rheb has been identified this year (Hsu et al., 2007). This regulator, named TCTP, appears to be important for Rheb activation in vivo and in vitro, and thus may represent a novel Guanine nucleotide Exchange Factor (GEF). Structure-function analysis of the Rheb-TCTP interaction is likely to provide important information about possible regulatory sites on Rheb. Based on the new data, rational small molecule drug design based on the three-dimensional structure of Rheb could be feasible.

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