Ion Channel Discovery & Methods

- Reading: None assigned, However, for your entertainment:
  - Ion channel structure/function: Kandel & Swartz
  - Molecular Biology techniques: I’ve taken many of my figures from “Molecular Cell Biology” by H. Lodish, et al.
  - Any decent cell biology or molecular biology textbook will have this same information. Two good ones are Genes VII (VIII?) by Lewin or “Molecular Biology of the Cell” by Alberts, et al.
  - Primary sources: These are all ground breaking papers, which I find interesting:
    - Papazian...Jan,LY. Cloning of genomic and complementary DNA from Shaker, a putative potassium channel gene from Drosophila. Science. 1987; 237:749-753.

To study the molecules, you have to identify the molecules

- Requires a pure preparation
  - Most enzymes can be assayed in pure preparations.
  - Ion channels assayed in living cells (usually).
- This requires a cDNA clone for expression.
- Ion channel proteins are of sufficient interest that every major methodology for cloning has been utilized.
Five Important Examples

- **Voltage-gated Na\(^+\) channels** from electric eels by purification & sequencing
- **Shaker K\(^+\) channels** by *Drosophila* genetics
- **Inward rectifying K\(^+\) channels** by expression cloning
- **HERG** by human genetics
- **Hyperpolarization-activated cation channel** by letting someone else do the work (database searching)

**Electrophorus electricus**

**Voltage-gated Na\(^+\) channel**

- To clone a gene product you need some information
  - Sequence information
  - Enough pure protein to produce antibodies
- Before large scale sequencing, you usually had to get this information directly from the protein.
- To purify a protein you usually need to fulfill two requirements
  - *A rich source*
  - *An assay*
Source: The electric eel

Why eel?

- Up to 2.5 meters long (1 to 1.5 meters more common)
- Electric organ makes up ~60-70% of the body length
- The organ is made up of thousands of plates containing a specialized muscle cells
- There are lots of Na⁺ channels
- How many?
  They can deliver a charge of 650 volts; 1 amp—enough to stun a horse.

Obviously a rich source of channels
A source is not enough, you need an assay

- Usually electrophysiological
- Alternative based on Tetrodotoxin (TTX) binding
- Isolated from organs of Puffer Fish (Fugu)
- Electrophysiological evidence showed that it was a potent & specific inhibitor of Na⁺ currents
- Can be labeled with $^{125}$I to provide an assay

Turning the $^{125}$I-labeled TTX into a method for detection of Na⁺ channels

*Diagram showing the process of labeling the TTX and measuring the labeled channels.*

Filter & Measure $^{125}$I from the filter
Now for the purification

- Isolate the membranes of eel’s electric organ
  - Homogenization
  - Centrifugation
- Dissolve the membranes with detergent
- Separate proteins by column chromatography
  - Size
  - Charge

Months (years?) of hard work produced

A single band on an SDS polyacrylamide gel

Why so broad?
How do you get to the cDNA?
Method 1: get the protein sequence

1. Digest with proteases
   - NH₂, COOH

2. Separate peptides
   - NH₂, COOH

3. Sequence by Edman degradation
   - NH₂

4. Prepare 20mer degenerate probe to screen genomic library

5. Perfectly complementary oligonucleotide in the degenerate probe

6. Coding sequence

What tissue would you use?

Use the oligonucleotide “probe” to screen a cDNA library

1. mRNA 5’ poly(A) tail
2. Transcribe RNA into cDNA
3. Remove RNA with salt
4. Hybridize with oligo-D primer
5. Synthesize complementary strand
6. Double-stranded cDNA
7. Protect cDNA by ethylation
8. Add poly(-dG) tail
9. Hybridize with oligo-C primer
10. Synthesize complementary strand
11. Protect cDNA by ethylation
12. Ligate cDNA to linkers
13. Cleave with EcoRI
14. Ligate to 3’ arms
15. Package in vitro infected E. coli
Screen cDNA library

How do you get to the cDNA?
Method 2: make antibodies

Inject pure protein into rabbit lymph nodes

Isolate IgG that will be rich in Anti Na+ Channel antibodies

Draw blood after several weeks
Antibodies require a different kind of library

- Library designed to express whatever reading frame is in the insert
- Express: mRNA → protein

Why use both?

This is where the antibody comes in

1. Incubate filter with radiolabeled secondary antibody
2. Perform autoradiography
3. Labeled secondary antibody
4. Primary antibody
   - Antibody identifies specific plaques
5. Fusion protein bound to nitrocellulose
6. Proteins bind to nitrocellulose
7. Remove filter
8. Overlay nitrocellulose filter
9. E.coli
   - In vitro packaging
   - Plate on bacterial lawn
10. E.coli
    - DNA
    - Fusion protein
    - Ligate
    - Digest with E.coli
    - lac promoter

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The result was a 1820 amino acid cDNA

1820 amino acids = about 200 kD

Now what: The dawn of structure/function studies

Hydropathy plot:

Numa noted the repeats, and based on this proposed:
Numa’s suggestion:

- He noted the charged region
- Suggested that it was the voltage sensor
- Failed to understand that it must cross the membrane to be able to sense voltage
- Bought into hydropathy

Cloning of the *Shaker* K⁺ channel from *Drosophila*

- Prior to *Shaker*, there were only three cloned channels (Na⁺, Ca²⁺, and ACh) all by of purification
- This approach is not possible for K⁺ channels
  - Lack of abundance source
  - No specific toxin ligand for assays.
**Shaker time-line**

- 1940s: *Shaker* phenotype first described (H. Luers)
- 1969: Kaplan & Trout re-isolated *Shaker* mutants with, three neurological phenotypes were found on separate alleles and could be distinguished under ether anesthesia:
  - *Shaker*
  - *Ether-a-go-go*
  - *Hyperkinetic (2)*

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**THE BEHAVIOR OF FOUR NEUROLOGICAL MUTANTS OF DROSOPHILA**

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**Shaker time-line**

- 1977: Lilly Jan found *Shaker* flies found to have abnormal synaptic transmission at neuromuscular junctions
- 1981: Flies found to be missing an A-type or transient outward K+ current
How did they know that *Shaker* mutants were K channels?

- Only the $I_A$ current was altered → not a general membrane defect
- Gene dosage → wild-type *Shaker* gene copy number proportional to amplitude of $I_A$

How did they know that *Shaker* mutants were K channels?

- Gating properties of *Shaker* mutants; different mutants could have different properties

| WT          | $Shk$ +/- (one gene missing) | $Shk$ kinetic (likely point mutation) |
Genetic methods allow cloning without sequence information

- Banding of *Drosophila* chromosomes
- Mutation X-linked
- Large library of X-chromosome deletions that show shaker phenotype.

Isolate clones from a genomic library: *in situ hybridization*

- Label individual clones from an X-chrom genomic library
- Hybridize to both shaker mutant & WT DNA
- Look for a difference
The moment of discovery

The probe that hybridizes to WT, but not mutant must contain the *Shaker* gene!

Isolation of the genomic clone

- Chromosomal *in situ’s*→approximate location for the gene
- Use clone 16F for chromosomal walk to isolate more clones spanning 210 Kb
Narrow down the area that contains coding portions of \textit{Shaker}

- Cut the genomic clones into smaller probes
- Perform Southern Hybridizations that compare mutant to wild type DNA to try to find coding regions

These experiments produced small (~2500 bp) pieces of DNA that were used to probe a cDNA library (made from mRNA) to get the sequence of the \textit{Shaker} protein
This process of successively narrowing finally gave the *Shaker* cDNA

There are lots of *Shakers*

- The discovery of one *Shaker* K channel cDNA allowed the much easier isolation of related cDNAs
Expression cloning of the inward rectifier $K^+$ channel (1993)

What is an inward rectifier?

- The current has been seen in many tissues:
  - Skeletal muscle, Heart, Brain (neurons & glia), Oocytes, Blood cells, Endothelial cells
- Up to this time, all cloned K channels were related to $Shaker$, yet none was an IR
- How to clone?
  - No apparent relationship to known channels
  - Purification?
    - Low abundance
    - No toxin
    - No mutants
This leaves us with expression cloning: *First, express the channel*

1. Isolate the mRNA of interest
2. Inject into a *Xenopus* oocyte

The mRNA’s will be translated into proteins

*If* the desired channel is expressed in sufficient amounts, the current can be detected by 2 electrode voltage clamp

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**How it really worked:**

- Isolated RNA from rat brain & skeletal muscle, rat & guinea-pig heart, cultured bovine aortic endothelial cells, plus 2 myoblast, pituitary, neuroendocrine & a macrophage cell line.
- 50 ng A⁺ mRNA injected into an oocyte
- RNA fractionated by size

Why so many tissues?
To isolate the clone

- Make a λ cDNA library from mRNA
- Transcribe RNA from the cDNA

Now isolate the clone by "Sib Selection"

$cDNA$ isolation by sib selection: $Dilute \rightarrow Amplify$

Start with a library

Red=channel
Gray=everything else

Sub-divide (dilute)

Repeat

Amplify

Rescreen & Dilute

PURE CLONE
**Result:** A clone they called IRK1

**Pitfalls of expression cloning**

- Why might the expression cloning fail?
  - All channels known, IF they look like a channel
  - Ancillary subunit

- Long QT syndrome is a condition characterized by a lengthening of the QT interval in an ECG
- Can result in serious or fatal cardiac arrhythmias
- It is normally acquired (often as a side effect of drug therapy), but in rare cases it is genetic
- It is autosomal dominant

Cloning a human gene responsible for an inherited disease: practical considerations

- Families that share a phenotype
- They need to be large, with at least 3 generations available
- They need to keep good records
- It is best if they share proximity & lifestyle
- With a subtle cardiac phenotype, they need to have had access to decent medical care and be willing to interact with physicians
Search for the region of the genome containing the mutation

- When a mutation is inherited, it is not just the mutation that is transmitted, but a huge portion of a chromosome.
- Only 1% of the genome has genes.
- The remainder has many features useful for mapping: **CA repeats**
- CA repeats
  - Are found every 40 kB or so on average (~90,000 total).
  - Their length varies, but is normally less than 200 bp.
  - Thousands have been mapped along the human genome.
  - *Just like any other trait, these repeats are inherited.*

CA repeats can help locate a mutant gene

- The trick is to find a CA repeat in a mutant individual(s) that is different in a wild-type (most will be the same because family members are compared).

Why are there two bands?

Why might there be only one?
Found a marker: DS7483

- Several candidate genes on chromosome 7
  - Chloride channel
  - Ca\(^{2+}\) channel
  - Poorly characterized K\(^+\) or cation channel (HERG)

Further mapping \textit{in situ} hybridization of the chromosome pointed to HERG

250,000 bp contained HERG gene
But if we knew about HERG all along, why wasn’t this found sooner?

- HERG: human *ether-à-go-go* related gene
- *Ether-à-go-go* discovered with *Skaker*.
- Distinct phenotype & not allelic
  - Funny name
  - Cloned in 1994, but with no EP
  - A grant to study the EP was submitted to NIH, and was in the reject pile
  - In fairness, it was a neurobiology study section. HERG gives an unusual and complex current; it was not recognized as heart $I_{Kr}$
  - Until Keating’s paper came out

Information from the genomic clones was used to isolate genomic DNA from mutant individuals

*These individuals carried several mutations in the HERG gene.*

- Electrophysiological analysis showed
  - HERG is responsible for an unusual K current found in the heart: $I_{Kr}$
  - The mutants were either non-functional or had decreased function
Let your fingers (computer) do the walking: Cloning of the Hyperpolarization-activated cation channel (1998)

- Important in the heart (involved in pace making) and in the nervous system (pacing of spontaneously firing neurons)
- The current was well studied in native heart & nervous tissue
- No disease, no mutant, no purification, how to clone?

Numerous attempts by expression cloning and low stringency hybridization (like the mammalian Shakers) failed

Hyperpolarization-activated cation channel

- This is what was known:
  - Stimulated by hormones and neurotransmitters that increase cAMP levels
  - This stimulation is not due to protein phosphorylation, so it is likely caused by direct binding of cAMP to the channel protein.
  - Blocked by Cs\(^+\) ions
Develop a Hypothesis

1. So you start with the hypothesis that you are looking for something that binds cAMP.

2. Fortunately, there are cAMP binding sites on several proteins, including cAMP-activated cation channels which are understood.

3. THEREFORE: Search GeneBank looking for things that “look like channels” that have cAMP binding sites, that are otherwise not known.

Searches the Genebank database for unassigned cyclic nucleotide binding sites

Go to the BLAST site

Search for cyclic nucleotide binding sites

Use the well-characterized cyclic nucleotide binding domain from the rod photoreceptor CNG cation channel

est=expressed sequence tags; cDNAs that have been picked randomly and partially sequence
BLAST Search

Then you wait.....

NCBI BLAST Search

Your request has been successfully submitted and put into the Blast Queue.

Query = (127 letters)

The request ID is P82885496-15031-14633

Not so bad: hundreds of users, billions of records

BLAST Results

Distribution of 84 Blast Hits on the Query Sequence
**BLAST Results**

A measure of the likely hood of a random match

**Match Significance**

**TOP:** 1/900000000000000000

**MIDDLE:** 1/62.5

**BOTTOM:** 3.4/1
Blast Results

The top turned out to be a mouse retinal CNG cation channel

That’s good!

At least you found what you expected, now how about something related

Blast Results

Mouse HPA cation channel!

Not 86.7 & 9×10⁻¹⁷, but close

Of course, at the time, they did not know this.
Data that would suggest that they have what they came for?

- Experimental:
  - Activated by hyperpolarization
  - Modulated directly by cAMP
  - Blocked by Cs+
- Other types of data?

It is easy to check the sequence

After all, that is how it was found in the first place

But ESTs don’t have the whole cDNA, just the 3’ or 5’ end. Plus they often contain errors

This one only has 478 nucleotides or at most 159 amino acids
It is easy to check the sequence

These folks sequenced it, and have it in the freezer. For a small fee, they’ll send it to you.

So you send away for the clone

• Complete the sequencing
• Use the clone to screen a cDNA library to see if there are any more like it & to be sure you have the complete cDNA.
• And try to figure out if it is a channel
  – Align with other channels
  – Look for channel features (an S6 just upstream of the CNB domain)
It looks like a channel with a cNT binding domain

It has 6 membrane spanning segments

It looks like a channel with a cNT binding domain

It has a cyclic nucleotide binding region just downstream from S6
It looks like a channel with a cNT binding domain.

It also has an S4 voltage-sensor and a pore region.

Electrophysiological evidence: It acts like a HPAC. Activated by hyperpolarization.
It Acts like a HPAC

Stimulated by cAMP

Blocked by Cs⁺

Is this the end?

- In 1974 almost no known DNA sequences
- Probably >50 x 10⁹ nucleotides
- If you remember one thing about my lectures,
  you’ll fail the test
- But: It should be that channels can be identified & understood by their families
  - We know the sequence features of channels
- This takes information
Genome projects (partial list)

Completed or underway
Aspergillus nidulans
Caenorhabditis elegans
Candida albicans
Candida tropicalis
Coccidioides immitis
Coprinus cinereus
Cryptococcus neoformans
Drosophila melanogaster
Human
Pneumocystis carinii
Pufferfish
Rat
Rhizopus oryzae
Roundworm
Sacccharomyces cerevisiae
Sea Squirt
Ustilago maydis
Snail, Freshwater
Beetle, Red Flour
Candida guilliermondii
Candida bantiana
Cat, Domestic
Chicken
Chimpanzee
Ciliate Oxytricha trifallax

Planned

Aspergillus nidulans
Caenorhabditis elegans
Candida albicans
Candida tropicalis
Coccidioides immitis
Coprinus cinereus
Cryptococcus neoformans
Drosophila melanogaster
Human
Pneumocystis carinii
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Rhizopus oryzae
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Candida guilliermondii
Candida bantiana
Cat, Domestic
Chicken
Chimpanzee
Ciliate Oxytricha trifallax

If we know what channels look like, we now know all the channels

- 88 $K^+$ channel genes in humans
- Probably exist as multiprotein complexes
- Know a lot, but not all
- How to find out?