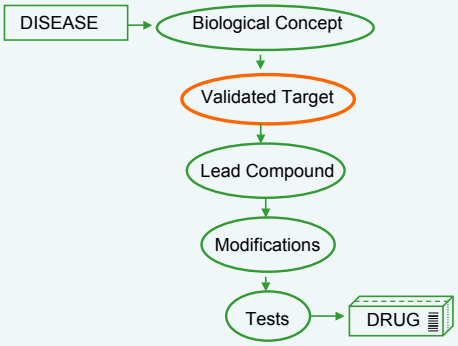
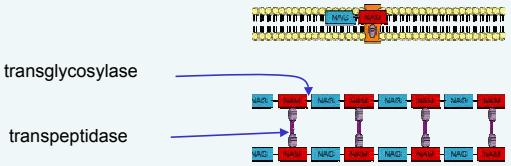
Current Approaches to Drug Discovery



What is a Target?

- Biological macromolecule or molecular complex that is critical for the disease
  - e.g. an enzyme that is <u>required</u> for the growth of the infecting bacterium



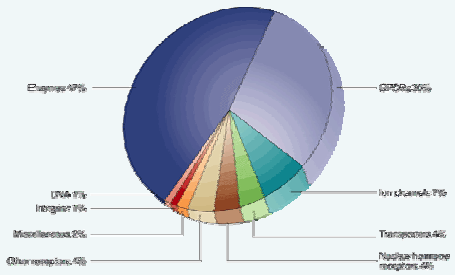
More: The alternative to penicillin
http://www.nature.com/cgi-taf/DynaPage.taf?file=/nm/journal/v7/n10/full/nm1001-1100.html

Drug Targets and Mechanism of Drug Action

TARGET	MECHANISM
Enzymes	Reversible & Irreversible Inhibitors
Receptors	Agonist and Antagonists
Viral Surface Proteins	Blocking the Entry to Cell
Ion Channels	Blockers and Openers
Transporters	Uptake Inhibitors or Enhancers
DNA, RNA	Alkylating Agents, Binders, Wrong Substrates (trojan horses)

Source: Hugo Kubinyi lecture slides. University of Heidelberg and BASF AG

Targets of Marketed Drugs



Hopkins and Groom: Nature Rev. Drug Discov. 1, 727-730 (2002)

Example Target: HIV Surface Proteins

Image: http://www.healthinitiative.org/html/hiv/firstcontact/hivbig.htm

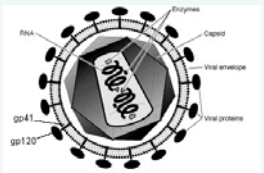


Image: http://www.accessexcellence.org/AB/GG/hiv.html

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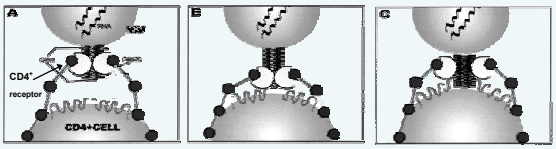
HIV: Virus-Cell Interaction



A: gp120 binds to $CD4^+$ receptor on the surface of T-cells

B: gp120 undergoes conformational change and binds to chemokine co-receptor

C: gp41 becomes exposed and forms a three-helix bundle

Image: www.medscape.com

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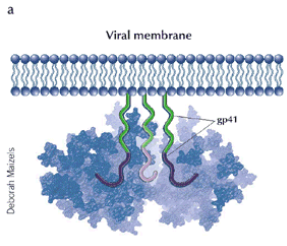
HIV gp41 and gp120



Structure before fusion

- gp41: viral fusion machinery
- gp41 is highly conserved
- gp120 is highly variable

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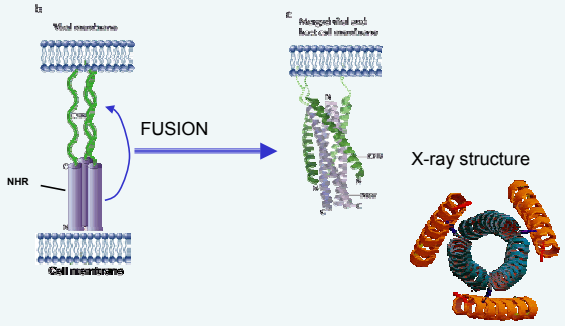
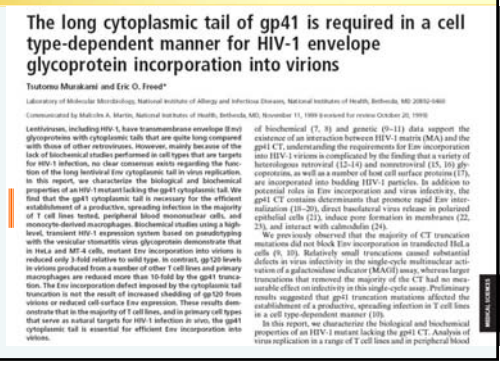
HIV gp41 Hairpin Formation Triggers Fusion



HIV gp41: Validation



The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions

Tsutomu Murakami and Eric O. Freed*

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892-0460

Communicated by Malcolm A. Martin, National Institutes of Health, Bethesda, MD, November 11, 1999 (received for review October 20, 1999)

Lentiviruses, including HIV-1, have transmembrane envelope (Env) glycoproteins with cytoplasmic tails that are quite long compared with those of other retroviruses. However, mainly because of the lack of biochemical studies performed in cell types that are targets for HIV-1 infection, no clear consensus exists regarding the function of the long lentiviral Env cytoplasmic tail in virus replication. In this report, we characterize the biological and biochemical properties of an HIV-1 mutant lacking the gp41 cytoplasmic tail. We find that the gp41 cytoplasmic tail is necessary for the efficient establishment of a productive, spreading infection in the majority of T cell lines tested, peripheral blood mononuclear cells, and monocyte-derived macrophages. Biochemical studies using a high-level, transient HIV-1 expression system based on pseudotyping with the vesicular stomatitis virus glycoprotein demonstrate that in HeLa and MT-4 cells, mutant Env incorporation into virions is reduced only 3-fold relative to wild type. In contrast, gp120 levels in virions produced from a number of other T cell lines and primary macrophages are reduced more than 10-fold by the gp41 truncation. The Env incorporation defect imposed by the cytoplasmic tail truncation is not the result of increased shedding of gp120 from virions or reduced cell-surface Env expression. These results demonstrate that in the majority of T cell lines, and in primary cell types that serve as natural targets for HIV-1 infection *in vivo*, the gp41 cytoplasmic tail is essential for efficient Env incorporation into virions.

of biochemical (7, 8) and genetic (9–11) data support the existence of an interaction between HIV-1 matrix (MA) and the gp41 CT, understanding the requirements for Env incorporation into HIV-1 virions is complicated by the finding that a variety of heterologous retroviral (12–14) and nonretroviral (15, 16) glycoproteins, as well as a number of host cell surface proteins (17), are incorporated into budding HIV-1 particles. In addition to potential roles in Env incorporation and virus infectivity, the gp41 CT contains determinants that promote rapid Env internalization (18–20), direct basolateral virus release in polarized epithelial cells (21), induce pore formation in membranes (22, 23), and interact with calmodulin (24).

We previously observed that the majority of CT truncation mutations did not block Env incorporation in transfected HeLa cells (9, 10). Relatively small truncations caused substantial defects in virus infectivity in the single-cycle multinuclear activation of a galactosidase indicator (MAGI) assay, whereas larger truncations that removed the majority of the CT had no measurable effect on infectivity in this single-cycle assay. Preliminary results suggested that gp41 truncation mutations affected the establishment of a productive, spreading infection in T cell lines in a cell type-dependent manner (10).

In this report, we characterize the biological and biochemical properties of an HIV-1 mutant lacking the gp41 CT. Analysis of virus replication in a range of T cell lines and in peripheral blood

PNAS (1997)
vol. 97
343–348

Target Validation

"Target validation is one of the most pressing problems in pharmaceutical R&D. Many industry experts believe that without additional well-validated targets, pharmaceutical companies are unlikely to be able to maintain current levels of profitability."

From the executive summary of Post-Genomic Target Validation: Next Generation Approaches and Tools for Optimizing Target Selection.

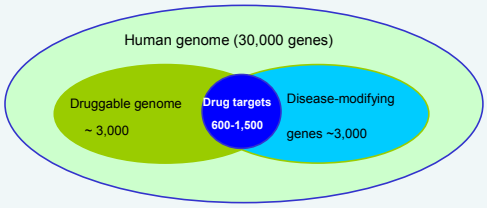
Validated Target

- Identification of a ***pathophysiologically relevant*** molecular target, e.g. an enzyme, receptor, ion channel, or transporter
- Determination of the DNA and protein sequence
- Elucidation of the function and mechanism of the protein
- Proof of the therapeutic concept in animals

Source: Hugo Kubinyi lecture slides. University of Heidelberg and BASF AG

Any Targets Left?

- Human genome: ca. 30,000 predicted genes
- Currently known targets: ca. 500



Target Validation Methods: Pre-genomic

- Goals:
  - Identify protein function
- Strategies:
  - Find out what does it interact with
  - Find out where in the cell it is
- Methods:
  - Systematic alteration of a gene
  - Phage display
  - Yeast-two-hybrid system
  - Expression cloning

Systematic Alteration of a Gene (Knock-out)

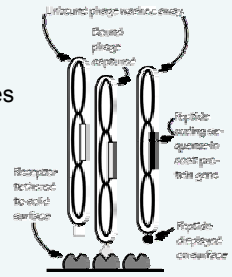
- Leishmaniasis (kala azar):
- Parasite infection
- 12 M infected worldwide

- Dihydrofolate reductase-thymidylate synthase deficient *Leishmania major* parasite does not cause disease

dUMP + 5,10-Methylene-THF $\xrightarrow{\text{TS}}$ **dTMP + Dihydrofolate**

Phage Display: Principle

- Screens for protein-protein interactions *in vitro*
- Living library in bacteriophages
- Unraveling signal-transduction pathways
- Hunt for viral surface proteins



More: http://www.biotech.missouri.edu/mbp/exchange/tips/3-99tips.html

Phage Display: Practice

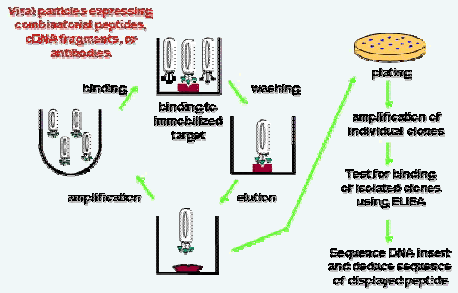
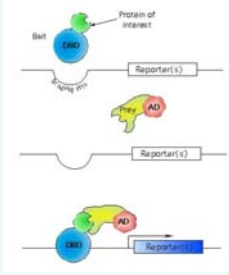


Image: http://www.bio.anl.gov/research/brian_kay.html

Yeast Two-hybrid System: Principle

- Screens for protein-protein interactions *in vivo*
- Living library in yeast
- Bait: Known protein fused to a DNA binding domain
- Prey: Protein from library fused to transcription activation domain
- Binding → Reporter gene ON



More: http://www.fccc.edu/research/labs/golemis/YTH_overview.html

Yeast Two-hybrid System: Practice

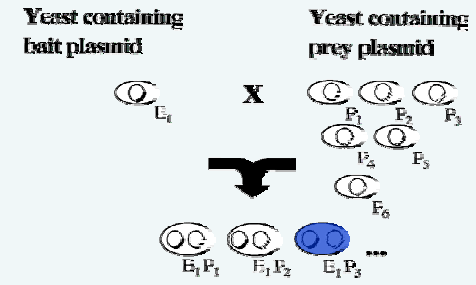


Image & More: Pierre Legrain*, Luc Selig

"*Genome-wide protein interaction maps using two-hybrid systems*" FEBS Letters 480 (2000) 32-36

Expression cloning

- Gene library in mammalian cells
- Identification of protein function by observing alterations in phenotype
- Identification of protein localization by monitoring protein-GFP fusions
- Function in normal environment
- Post-translational modifications

More: http://www.dkfz-heidelberg.de/LIFEdb/Cloning/strategy.html

Post-genomics methods: Identifying genes

- Goals:
  - Identify gene/protein function
  - Identify disease-modifying genes
- Strategies
  - Take advantage of human genome sequence
  - High-throughput screening methods
  - Automation, robotics, microfluidics, bioinformatics
- Methods:
  - DNA Microarrays
  - Single Nucleotide Polymorphisms
  - Proteomics with MS sequencing
  - RNA knockdown: antisense, ribozymes, RNAi

Is it Important?

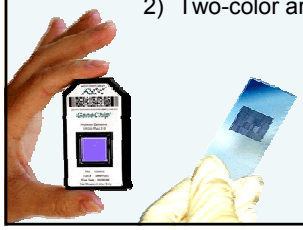
Published in September 2003
by Cambridge Healthtech,
128 pages.

•Print **$2,500.00**
•Single-site license **$5,000.00**
•Enterprisewide license **$7,500.00**

DNA Microarrays

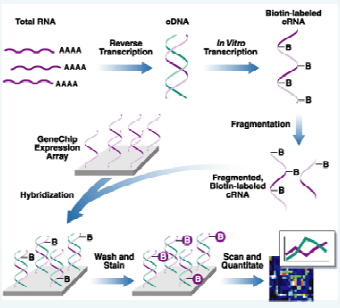
Two fundamental approaches

1) One-color array (Affymetrix)
2) Two-color array (Stanford, 1996)



Images and more:
http://www.technologiesnews.net/news.html?view=204
http://www.dhgp.de/ethics/ethics02.html

One-color Quantitative Microarray technology

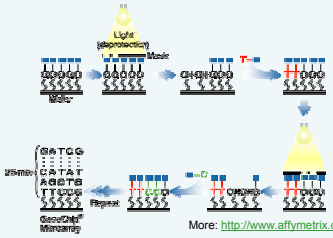


- Affymetrix GeneChip®
- Target is a biotin-labeled cRNA
- Probe is a single-stranded DNA oligo attached to the wafer
- Complementary target and probe hybridize
- Duplex is stained with the streptavidin-bound fluorescent marker

http://www.affymetrix.com/technology/ge_analysis/index.affx

GeneChip® Technology

- In situ oligonucleotide synthesis (Affymetrix U.S. patent 5,861,242)
  - 5-inch square quartz wafer with covalently bound layer of silane
  - Parallel synthesis with photolithographic masks
  - Makes oligos 20-25 nucleotides long; 400,000 per chip



More: http://www.affymetrix.com/technology/manufacturing/index.affx
http://www.devicelink.com/ivdt/archive/98/09/009.html

Biotinylated cRNA Targets

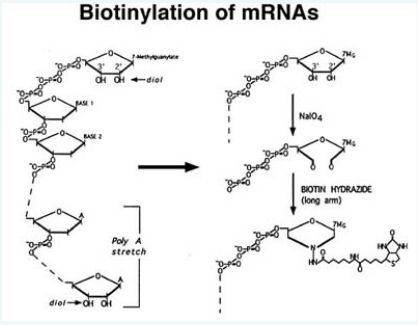
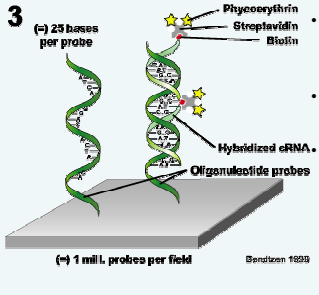
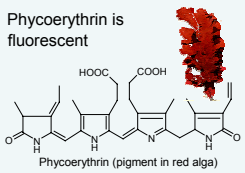


Image and more: http://pgec-genome.ars.usda.gov/FUNCTIONAL_DIR/descrStrategy.html

GeneChip®: Detection



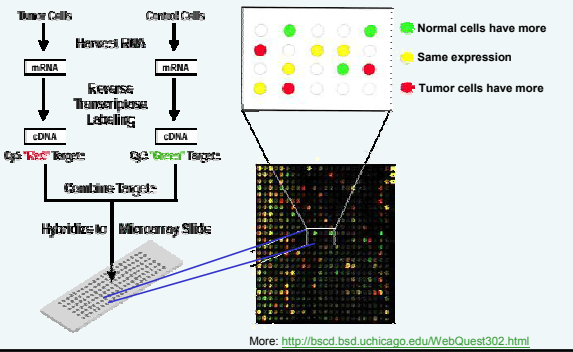
- Bound nucleic acid duplexes have a covalently attached biotin
- Biotin binds a streptavidin-phycoerythrin complex
- Phycoerythrin is fluorescent



Phycoerythrin (pigment in red alga)

More: http://home20.inet.tele.dk/iir/IIR/15genes/geneChip1.htm

Two-Color DNA Microarray "Genome Chips"

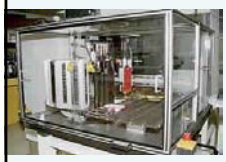


More: http://bscd.bsd.uchicago.edu/WebQuest302.html

Genome Chips: General Applications

DNA Microarrays allow simultaneous screening of many thousands of genes: high-throughput screening

- genome wide genotyping
  - Which genes are present in this individual?
- tissue-specific gene expression
  - Which genes are used to make proteins?
- mutational analysis
  - Which genes have been mutated?



Gene Machines OmniGrid™ Microarraying Robot at Penn State
http://hils.psu.edu/stf/dnama/Instrumentation.html

Genome Chips: Pharmaceutical Applications

Drug discovery:

- expression analysis in different disease states
  - identify differently expressed genes
- single nucleotide polymorphism analysis
  - identify genes in inheritable diseases
- pharmacogenomics
  - predict efficacy in a selected patient population
- toxicogenomics
  - predict toxicity in a selected patient population
- identification of bacteria in a mixed population

More: http://www.bri.nrc-cnrc.gc.ca/pdf/Microarray-Chapter.pdf

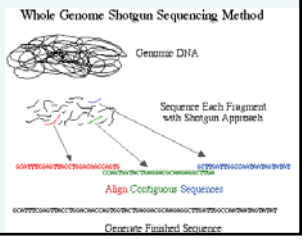
Single Nucleotide Polymorphisms (SNP)

- SNPs are the most important and basic form of variation in the genome
- The variation occurs in both coding and noncoding sequences
- Frequency of ca. 1 per 1000 base pairs
- About 1.8 M SNPs characterized
- SNP Account for the vast majority of polymorphism responsible for human disease

Image and More: http://www.bio-itworld.com/archive/090902/pharma.html

Finding SNP

- Shotgun sequencing of genomes of 24 different individuals
- Comparison of overlap regions between sequenced regions in the Human Genome Project



SNP Detection

- Single-stranded oligonucleotide probe
- Forms a stem-and-loop structure
- The loop contains a probe sequence
- A fluorophore is linked to the end of one arm
- A quencher is linked to the end of the other arm

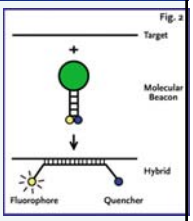


Image and More: http://www.molecular-beacons.com

Genotyping by SNP

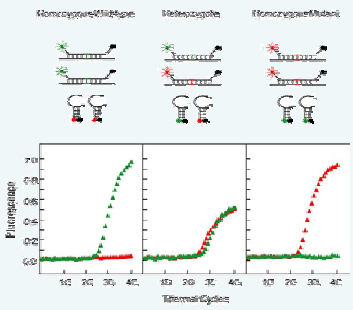


Image and More: http://www.molecular-beacons.com/Introduction.html

SNP Applications

- Identification of disease-causing genes by:
  a) Screening of families with inherited diseases
  b) Performing large-scale population studies
- Disease genes or chromosomal loci found:
  - Crohn's disease: NOD2/CARD15 gene in cr.15
  - Parkinson's: PARK10 in cr.1
  - Stroke: STRK1 in cr.5 (Islandic population study)
  - Type-2 Diabetes: (PPAR-γ 2 Nuclear Receptor)
- Identification of individuals with disease-causing genes
- On-Line database: http://snp.cshl.org/

Proteomics: "Genes were Easy"

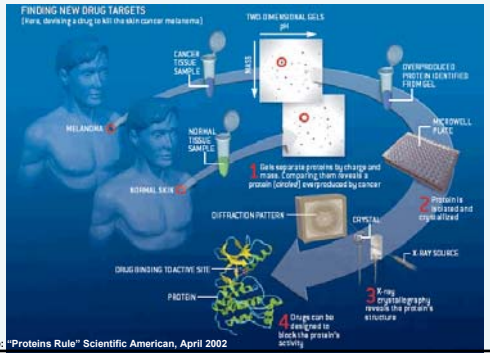


Image: "Proteins Rule" Scientific American, April 2002

Proteomics Methods

- 2D Electrophoresis
- Mass spectrometry
- Cell imaging
- Protein chips
- Protein crystallography
- Protein structure prediction

Image: www.infosci.coh.org/corelab/ proteomics.htm

Post-genomics methods: functional genomics

Testing protein function by mRNA knockdown

- Antisense Oligonucleotides
- Ribozymes
- RNA Interference

Antisense Oligonucleotides

- Suppression of the gene expression
- Allows to study the gene function

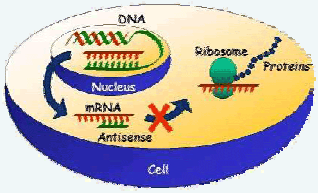
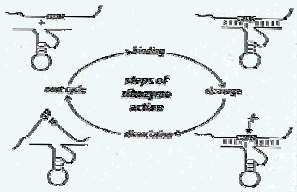


Image and more: http://perso.club-internet.fr/ajetudes/nano/antisense.htm

Ribozymes

- Catalytic RNA molecules
- Cleave mRNA sequence-specifically
- Can be prepared synthetically



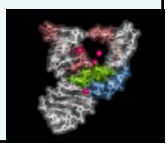


Image & more: http://academic.brooklyn.cuny.edu/chem/zhuang/QD/toppage1.htm
http://www.chem.ucsb.edu/~molvisual

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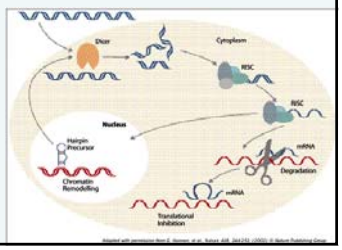
RNA Interference (RNAi)

- "RNA Immune System"
- ds RNA activates a process by which mRNA complimentary to ds RNA is degraded
- Discovered in 1998
- Gene function discovery
- Possible therapeutic value
- Systemic RNAi in plants



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RNAi: Pathway

- Dicer: endoribonuclease that cuts ds RNA into small pieces
- RISC: nuclease that uses siRNA as a guide to cleave mRNA

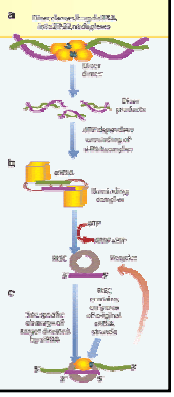

Image: Nature Biotechnology, 21 (2003), 1457-1465

RNAi:
Introduction of siRNA and shRNA

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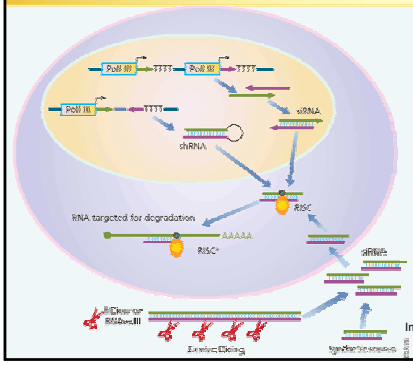

Image: Nature Biotechnology, 21 (2003), 1457-1465

Thank You!

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