CCR frontiers IN SCIENCE

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From the Director

CCR Core Facilities: Evolving to Reflect Institutional Priorities and Better Meet the Needs of Intramural Investigators

Core facilities within the CCR not only provide state-of-the-art technologies and other scientific resources to intramural investigators, but also disseminate expertise throughout the community through one-on-one consultation and a variety of training programs. They are loci for collaborative support and provide an infrastructure that stimulates interdisciplinary exchange. Their functions, moreover, continue to evolve as the state of the science advances, the needs of researchers change, and the CCR's priorities become redefined. Many cores are now taking on new roles as facilitators of translational research, as centers for advanced technology development, and as conduits for partnerships between intramural scientists and representatives of other government agencies, academia, and private industry.

A broad range of core scientific expertise exists within the CCR and within the Research Technology and the Clinical Services programs (RTP and CSP) operated by SAIC. The CCR, RTP, and CSP core facilities available to intramural researchers can be grouped into seven broad categories: animal sciences; clinical research and trials support; genetics and genomics; protein analysis and proteomics; analytical chemistry; cell analysis; and biomedical imaging. Examples of facilities devoted to animal sciences include those dedicated to technical services such as diagnostics, cryopreservation, and assisted reproduction, as well as several devoted to developing animal models, including the Transgenic Mouse Model Laboratory, the Germline Mutation Core, and the Gene Targeting Facility belonging to the Mouse Cancer Genetics Program. Core facilities supporting clinical research and trials include the Tissue Array Research Program, the Laser Capture Microdissection Core, and the SAIC Clinical Services Program. More information about CCR core facilities can be found at http://ccr.nci.nih.gov/resources/core_facilities.asp.

The number of core facilities providing services supporting research in genetics and genomics is especially large, including two microarray laboratories, the Laboratory of Molecular Technology, the DNA Sequencing MiniCore, and the Core Genotyping Facility of the Division of Cancer Epidemiology and Genetics, to which CCR investigators have access. Analytical chemistry cores include the Mass Spectrometry Center, the Separations Technology Group, and two nuclear magnetic resonance facilities. Facilities supporting proteomics research include two protein expression core laboratories, the Structural Biophysics Laboratory, and two protein chemistry cores. Cores devoted to cell analysis include flow cytometry laboratories belonging to various branches and the Comparative Molecular Cytogenetics Core. Biomedical imaging cores include facilities supporting confocal microscopy, the Image Analysis Laboratory, and the Fluorescence Imaging Facility. Finally, the Advanced Biomedical Computing Center provides support not only to individual investigators but also to other core facilities.

Overseeing the continued evolution of core facilities is a primary responsibility of the Office of Science and Technology Partnerships (OSTP) headed by David J. Goldstein, Ph.D. To accomplish its mission of providing

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CCR investigators with access to the most advanced technologies available, this office not only manages CCR and RTP cores, but also promotes scientific collaborations aimed at developing new technologies, scouts for emerging technologies, and informs the community about the novel opportunities such technologies afford.

The OSTP consults with CCR Principal Investigators, Faculties, the Technology and Translational/Clinical Initiatives steering committees, RTP scientists, and core facility managers to assess research needs and then identifies extramural organizations developing technologies that can fulfill those needs. It also provides a central point of contact for companies wanting to have newly developed technologies beta tested, refined, and validated by CCR investigators. Working with Dinah S. Singer, Ph.D., Director of the Division of Cancer Biology, and the NCI Technology Transfer Branch, the OSTP is now exploring the feasibility of establishing an independent beta-testing core laboratory. The office also helps formalize partnerships between CCR scientists and outside organizations, liaising with the NCI's Technology Transfer Branch and the NCI Office of the Director. Thus, the OSTP acts as a two-way bridge linking CCR investigators with their extramural counterparts.

The OSTP also supports the development of collaborative relationships among core laboratories. These collaborations are beginning to yield significant payoffs. To cite a few examples, the heads of five confocal microscopy cores—Stephen Lockett, Ph.D., James McNally, Ph.D., Susan Garfield, M.S., Sriram Subramaniam, Ph.D., and Michael Kruhlak, Ph.D.—are working together to develop an innovative software package in collaboration with the NIH Center for Information Technology. This software will assist researchers in extracting quantitative information from biomedical images and may become a common image-analysis and visualization platform across NCI/NIH microscopy laboratories, thereby facilitating inter-laboratory collaboration. The CCR's two complementary microarray core facilities—the Microarray Laboratory, which is part of the RTP's Laboratory of Molecular Technology headed by David Munroe, Ph.D., and the Microarray Facility headed by Ernest Kawasaki, Ph.D., which is part of the Advanced Technology Center in Gaithersburg—are now jointly evaluating oligonucleotide arrays in anticipation of the expected transition away from cDNA-based to oligo-based technology. Finally, the Gene Targeting Facility of the Mouse Cancer Genetics Program not only provides support to investigators generating gene-targeted mutations in mice, but also works closely with RTP core laboratories to develop the technologies needed to characterize the phenotypes and genotypes of such models. This close integration of animal science with advanced technology development is critical to the success of the NCI's animal models initiatives.

While ad hoc collaborations among core facilities continue, the OSTP also supports the development of more formalized collaborative infrastructures through the creation of what are, in essence, "meta cores." Most striking in terms of its long-term implications for translational research is the emergence of the Biomedical Proteomics Program (BPP) under the directorship of Timothy Veenstra, Ph.D. The BPP draws expertise from six RTP core laboratories—the Protein Chemistry Laboratory, the Protein Expression Laboratory, the Laboratory of Molecular Technology, the Image Analysis Laboratory, the Mass Spectrometry Center, and the Advanced Biomedical Computing Center—to identify and characterize proteins playing key roles in carcinogenesis and disease progression. Investigators in these laboratories are now working synergistically to offer expertise that extends from sample acquisition, sample handling, separation technologies, and the generation of affinity reagents to protein discovery and analysis of post-translational protein modifications through mass spectrometry-based techniques.

Most striking in terms of its long-term implications for translational research is the emergence of the Biomedical Proteomics Program.

Along with offering services and expertise to investigators throughout the CCR, the BPP collaborates closely with the Clinical Proteomics Program (CPP) headed by Lance Liotta, Ph.D., and Emanuel Petricoin, Ph.D. The unique collaboration between the BPP and the CPP allows information to flow simultaneously from the bench to the bedside and from the bedside to the bench. The CPP, for example, provides the BPP with clinical samples purified through its Laser Capture Microdissection Core Laboratory, while the BPP provides the analytical tools necessary to elucidate the proteins characterizing clinical samples. The major goal of this iterative process is to translate the discoveries made possible by the new science of proteomics into diagnostic, prognostic, and therapeutic applications as quickly as possible. The BPP and CPP are now setting up a Clinical Proteomics Reference Laboratory to accelerate the FDA approval process and make proteomics-based diagnostic tools quickly available to the clinical community.

The scale of the BPP, CPP, and the Clinical Proteomics Reference Laboratory underscores the CCR's exceptionally strong commitment to expediting the translation of basic discoveries into the clinical setting. The synergism among these three entities, moreover, not only offers an intriguing glimpse into the future evolution of the CCR's research infrastructure, but also augurs the future direction of cancer research in general.

To learn more about CCR core facilities, visit http://ccr.nci.nih.gov/resources/core_facilities.asp, where a full listing of core facilities, along with information on how to access their services, is available.

■ Carl Barrett, Ph.D.

■ CLINICAL RESEARCH

The Promise of Proteomics

Investigators in the CCR have played an extensive role in getting proteomics on the map. Proteomics—the study of proteins inside cells—is generating substantial media and scientific interest. The excitement of proteomic technology for scientists is that patterns of serum proteins might reflect underlying pathology and therefore serve as diagnostic or prognostic biomarkers. Scientists are interested in the potential of proteomics to detect cancer early, predict recurrence, and elucidate how a drug works.

Researchers in the FDA/NIH Clinical Proteomics Program reported in a recent study that a simple blood test might be able to detect ovarian cancer, even in its earliest stages. Elise Kohn, M.D., Lance Liotta, M.D., Ph.D., and Seth Steinberg, Ph.D., served as NCI investigators on the study working with Emanuel (Chip) Petricoin, Ph.D., of CBER, FDA.

The study, which was published online and in print in the *Lancet* earlier this year (Petricoin EF, et al. *Lancet* 359: 572-7, 2002), found that protein patterns in blood serum may distinguish women with ovarian cancer from women without the disease. First, the researchers studied a learning set of serum samples from women known to have ovarian cancer and from unaffected women. They identified a combination of five proteins present at different levels in the two groups of women. With this pattern, the investigators were able to correctly identify 50 out of 50 women with cancer and 63 out of 66 women without cancer from a second, blinded, test sample set. A major finding was the test's ability to detect all cases of stage 1 ovarian cancer.

Current research focuses on validating these findings by using extant collections of patient serum samples. In addition, two other proteomics clinical trials are under way. The first is designed to determine whether proteomic technology can reliably detect relapse of ovarian cancer before symptoms occur. The second is testing whether a patient's clinical response to the drug Gleevec™ can be correlated with the protein changes observed at a molecular level; this trial will be the first to evaluate Gleevec™ as a potential treatment for ovarian cancer. Dr. Kohn is the lead investigator for both studies.

To obtain additional information about proteomics or the CCR studies under way, contact the Clinical Studies Support Center at 1-888-NCI-1937.

The Importance of Mucosal Cytotoxic T Lymphocytes in Control of SIV/HIV Infection and AIDS-like Disease in Primates

Belyakov IM, Hel Z, Kelsall B, Kuznetsov VA, Ahler JD, Nacsa J, Watkins DI, Allen TM, Sette A, Altman J, Woodward R, Markham PD, Clements JD, Franchini G, Strober W, and Berzofsky JA. Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. *Nat Med* 7: 1320-6, 2001.

Human immunodeficiency virus (HIV) and its close cousin, simian immunodeficiency virus (SIV), are primarily transmitted through genital or gastrointestinal mucosal surfaces. The gut mucosa, which contains more potentially infectable $CD4^+$ T lymphocytes than all the other lymphoid organs combined, has been identified as a major reservoir for virus infection and replication. An appropriate immune response in this compartment may thus be critical to control HIV or SIV infection once it occurs. $CD8^+$ cytotoxic T lymphocytes (CTL) are necessary to control SIV infection in Rhesus macaques, and circumstantial evidence indicates that the same is true for HIV. An HIV vaccine should therefore aim to induce CTL in the mucosal tissues. However, most AIDS vaccine studies have not focused on mucosal routes of immunization, and only some have studied a mucosal route-of-challenge infection.

In collaboration with investigators at NCI, the National Institute of Allergy and Infectious Diseases, the National Institute of Child Health and Human Development, and other institutions, Igor M. Belyakov, M.D., Ph.D., D.Sc., and Jay A. Berzofsky, M.D., Ph.D. (Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch), investigated the ability of vaccines to induce mucosal CTL and to ultimately prevent viral transmission across a mucosal barrier. They also studied the effect of mucosal CTL on SIV infection. Initial studies in mice showed that an intrarectal route of immunization with an HIV peptide vaccine was highly effective at inducing CTL both in mucosal tissues and in systemic tissues such as the spleen, thereby protecting against a recombinant vaccinia virus expressing the HIV envelope protein. On the other hand, a subcutaneous route of immunization induced CTL mostly in the systemic compartment (Belyakov IM, et al. *Proc Natl Acad Sci U S A* 95: 1709-14, 1998). Further studies showed that vaccine protection depended on $CD8^+$ CTL to be present locally in the mucosa (Belyakov IM, et al. *J Clin Invest* 102: 2072-81, 1998). The presence of CTL in the systemic compartment was not sufficient for vaccine-induced protection.

In the current study, Drs. Belyakov and Berzofsky and their collaborators have translated this research to a non-human primate model in which Rhesus macaques are infected with a pathogenic, HIV-like retrovirus that causes an AIDS-like illness. They have compared mucosal and systemic routes of immunization, using a peptide vaccine to avoid a replicating virus that might spread beyond the site of immunization. The vaccine contains helper T-cell epitopes from HIV that are immunogenic in mice, humans, and monkeys (Berzofsky JA, et al. *J Clin Invest* 88: 876-84, 1991). Instead of carrying the HIV CTL epitope used in mice, however, the vaccine contains CTL epitopes from the SIV gag and pol proteins, which have been shown to be presented by the monkey major histocompatibility (MHC) molecule, Mamu-A*01. Mamu-A*01–positive monkeys were selected to ensure a response to the CTL epitopes.

The animals were organized into three groups: one received the mixture of vaccine peptides intrarectally; another group received the same peptides subcutaneously; and the control group received the mucosal adjuvant, without peptides, intrarectally. After two cycles of four immunizations, both the subcutaneous and intrarectal groups generated CTL at varying levels, as detected in colonic biopsies and mesenteric lymph nodes from the mucosal group and in the axillary lymph nodes from the subcutaneous group. None of the control animals exhibited detectable CTL in any site tested. The level of CTL activity correlated significantly with the level of helper T-cell response. This finding is consistent with the fact that although the animals were selected for the Mamu-A*01 class I MHC molecule, they were outbred for the class II MHC molecules that determine the helper T-cell responses, accounting for the variation in T-cell help and thus indirectly for the variation in CTL response. The result suggests that in primates, as has been shown in mice, the ability of a vaccine to induce CTL depends highly on its ability to induce helper T cells.

Two weeks after the final boost, the monkeys were challenged intrarectally with 10 animal-infectious doses of SHIV-ku2. This virus is a pathogenic strain of SHIV, a chimeric virus expressing the envelope of HIV with the gag and pol proteins of SIV. This virus thereby expresses all the epitopes contained in the vaccine. The majority of the animals in all three groups showed high peaks of virus in the blood, indicating that the CTL induced by the vaccine were not sufficient to prevent transmission. However, when the viremia tapered off to a plateau or "set point," it was noted that all the animals immunized mucosally had set points below the limit of detection (2,000 copies/ml), whereas those immunized subcutaneously and those in the control group had substantially higher set points. From 63 to 196 days after challenge, the differences in the set

The result suggests that in primates, as has been shown in mice, the ability of a vaccine to induce CTL depends highly on its ability to induce helper T cells.

points between the mucosally immunized group and the subcutaneously immunized group remained statistically significant. Thus, mucosal immunization had a favorable impact. Consistent with this finding, animals immunized mucosally exhibited a higher CD4 cell count and fewer opportunistic infections than those immunized subcutaneously.

This difference, however, was surprising. Because mucosal immunization was not sufficient to reduce mucosal transmission in these animals, the higher efficacy of this route of immunization on viral load in the bloodstream, compared with systemic immunization, was not expected. Drs. Belyakov and Berzofsky reasoned that if the gut mucosa represented a major site of virus replication, and if this site were one of the primary reservoirs seeding the bloodstream with virus, then mucosal CTL might be more effective at clearing this reservoir, thus reducing the level of virus seeded into the bloodstream. Postmortem examination of the animals' gastrointestinal tracts, approximately 200 days after infection, showed that CTL activity, without *in vitro* stimulation, was greater in the colons of the mucosally immunized group than in those from the other groups, even though all were infected with virus. Conversely, the levels of virus in the colonic and jejunal tissue samples from the mucosally immunized animals were all below the limit of detection, whereas the levels in these tissues from the subcutaneously immunized and control groups were 10- to 100-fold higher. These results strongly support the hypothesis that the mucosal route of immunization induced more CTL in the gut mucosa, thereby eradicating more effectively the virus from this major reservoir, which was seeding the bloodstream.

Overall, the results of these studies suggest that the vaccine-mediated induction of CTL in the mucosal tissues may be critical for an effective AIDS vaccine, not only to potentially prevent or reduce transmission across the mucosal barrier, but also to more effectively control the virus by eradicating it from a major site of virus replication. The investigators are now extending this work to develop an improved mucosal vaccine that will induce higher levels of CTL to prevent or reduce mucosal transmission of SHIV and control any infection that does occur. The lessons from these studies provide an important guide to the development of effective AIDS vaccines.

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■ CANCER AND CELL BIOLOGY

Human Mitochondrial Topoisomerase I: A Missing Close Relative Found

Zhang H, Barceló JM, Lee B, Kohlhagen G, Zimonjic DB, Popescu NC, and Pommier Y. Human mitochondrial topoisomerase I. *Proc Natl Acad Sci U S A* 98: 10608-13, 2001.

DNA topoisomerases modify DNA topology by introducing reversible breaks in the DNA phosphodiester backbone. These enzymes are ubiquitous and essential for DNA strand separation, relaxation of DNA supercoiling, chromatin compaction, and DNA decatenation during transcription, replication, and recombination. DNA topoisomerases are classified into two types, based on their enzymatic DNA intermediates. Intermediates of the type I class consist of single-stranded DNA breaks. Type I topoisomerases are further categorized into type IA and type IB enzymes. Type IA enzymes, such as top3α and top3ß, break the DNA by forming a covalent bond to the 5' end of the broken DNA, whereas type IB topoisomerases, such as top1, bind covalently to the 3' end of the break. Type II topoisomerases function as dimers and form staggered, double-stranded DNA breaks with one enzyme molecule bound to each of the 5' ends.

The mitochondrial genome consists of a circular duplex molecule of 16,659 base pairs. Each cell contains more than 1,000 mitochondrial genome molecules distributed into 2 to 10 copies per mitochondrion. Because the mitochondrial genome has to be transcribed, replicated, and repaired, mitochondrial topoisomerase activities are needed. Following years of confusing results about the existence of a separate mitochondrial DNA topoisomerase I, we were able to

*Following years of confusing results about the existence of a separate mitochondrial DNA topoisomerase I, we were able to identify the mitochondrial top1 gene (*TOP1mt*).*

identify the mitochondrial top1 gene (*TOP1mt*). Until now only one topoisomerase I gene had been identified in human cells. Because the mitochondrial topoisomerase protein occurs in low abundance, efforts to sequence the corresponding peptide were hampered, precluding the identification of the *TOP1mt* gene.

We conducted a BLAST search for new topoisomerases and found a novel human top1 cDNA sequence. We cloned this cDNA and expressed it as a recombinant polypeptide. Using green fluorescence protein (GFP)-tagged polypeptides, we showed that the recombinant protein localized to mitochondria. This discovery puts to rest any question of whether nuclear topoisomerases are imported into the mitochondria to resolve the topological problems attendant with the replication, repair, recombination, and expression of the mitochondrial genome.

The human *TOP1mt* gene (GeneBank accession no: AF349017) is a nuclear gene rather than one of the 13 genes encoded by the mitochondrial genome, as is the case for other genes encoding mitochondrial proteins. Cytogenetic analyses demonstrated that *TOP1mt* is on chromosome 8q24.3. Sequence comparison with the nuclear top1 peptide indicates a very high degree of conservation between top1mt and nuclear top1 in the core and C-terminal domains (Figure 1). The last 13 exons of both genes also exhibit identical size and intron boundaries, suggesting that *TOP1mt* is not derived from any of the prokaryotic *TOP1* genes, which are very different from their eukaryotic counterparts, but from a partial duplication of nuclear *TOP1*. The mitochondrial localization of the top1mt protein is controlled by its N-terminal domain, which contains a mitochondrial targeting sequence. The corresponding region of nuclear top1 contains nuclear localization sequences (Figure 1). The mitochondrial and nuclear top1 enzymes differ by their biochemical requirements. Top1mt requires high pH (around 8) and divalent cations (Ca^{2+} or Mg^{2+}) for optimal catalytic activity, whereas nuclear top1 has the optimal pH of 7.2 with less stimulating effect from divalent cations.

Like nuclear top1, top1mt is a type IB enzyme that is sensitive to camptothecin, a potent anticancer drug. Camptothecin becomes a cellular poison by inhibiting the re-ligation step of top1 catalysis. As a result, single-stranded DNA breaks are generated at the sites of DNA cleavage, and these can become double-stranded DNA breaks if there is a collision with a replication or transcription fork. The sensitivity of top1mt to this drug may extend the list of the known cellular effects of camptothecin and its derivatives.

It is not known whether any human genetic disorders are associated with *TOP1mt* gene alterations. The expression levels of the *TOPmt* gene may regulate mitochondrial DNA copy number, and a loss of expression of top1mt may predispose persons to mitochondrial diseases with neurodegenerative and myopathy components or to late-onset neurodegenerative diseases. Animal models harboring a conditional knockout for this gene may further define the role of *TOP1mt* in the topology of the mitochondrial genome and in disease.

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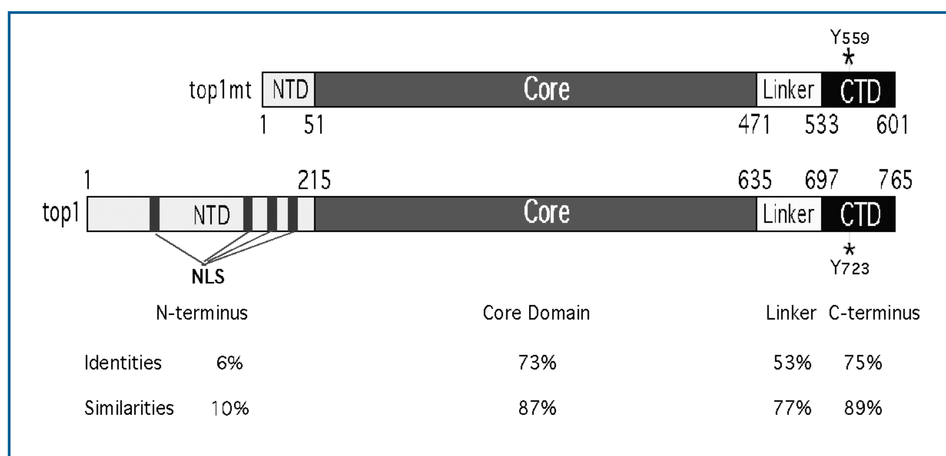

Figure 1. Sequence comparison of mitochondrial (top) and nuclear (bottom) top1 peptides. NLS, nuclear localization sequence; NTD; N-terminus domain; CTD, C-terminus domain.

■ TRANSLATIONAL RESEARCH

Improved Cytotoxic Activity of a Mutant Anti-CD22 Immunotoxin

Salvatore G, Beers R, Margulies I, Kreitman RJ, and Pastan I. Improved cytotoxic activity toward cell lines and fresh leukemia cells of a mutant anti-CD22 immunotoxin obtained by antibody phage display. *Clin Cancer Res* 8: 995-1002, 2002.

In 2001, Robert Kreitman, M.D., and coworkers (Laboratory of Molecular Biology) reported the development of a recombinant immunotoxin that showed considerable promise in treating certain B-lymphocyte malignancies (Kreitman R, et al. *N Engl J Med* 345: 241-7, 2001). This immunotoxin consists of the variable portion of the RFB4 antibody—which recognizes the CD22 marker on B lymphocytes—coupled to the PE38 portion of *Pseudomonas* exotoxin. In clinical trials, the immunotoxin promoted complete remission in 11 of 16 patients with chemotherapy-resistant hairy cell leukemia.

To improve the efficacy of the immunotoxin for this and other leukemias, Giuliana Salvatore, Ph.D., Richard Beers, B.S., Dr. Kreitman, and Ira Pastan, M.D., used a phage display library of mutants to increase the affinity for CD22. Efforts focused on a mutational hot spot within the 14-residue third complementarity determining region of the immunoglobulin heavy chain, because a major portion of the immunotoxin's binding specificity depends on this region. A sequence of four consecutive amino acid residues, GSSY, was randomly mutated to produce a phage display library of 160,000 clones, which were screened for improved binding to cells expressing the native CD22. The mutant sequence GTHW increased the binding affinity 14-fold. The resulting immunotoxin was 5- to 10-fold more active on various CD22-positive cell lines and up to 50-fold more cytotoxic to cells from patients with chronic lymphocytic leukemia (CLL) and hairy cell leukemia.

These results demonstrate the value of targeting hot spots to increase the affinity of antibodies. The new, higher-affinity immunotoxin developed through this mutational process has several potential major advantages. Among those advantages are efficacy at lower doses, where fewer side effects from the toxin moiety would be expected, as well as efficacy against leukemic cells that have lower densities of CD22, as in the case of CLL.

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■ MOLECULAR BIOLOGY

New Molecular Targets for Cancer Prevention: Genes Transcriptionally Regulated by Fra-1

Young MR, Nair R, Bucheimer N, Tulsian P, Brown N, Chapp C, Hsu T-C, and Colburn NH. Transactivation of Fra-1 and consequent activation of AP-1 occur extracellular signal-regulated kinase dependently. *Mol Cell Biol* 22: 587-98, 2002.

Because the rate-limiting events in multistage carcinogenesis occur during tumor promotion and subsequent tumor progression, discovery of the molecular events that drive tumor promotion can reveal important molecular targets for cancer prevention. Activation of activator protein 1 (AP-1)–dependent transcription is required for neoplastic transformation by tumor promoters in the mouse JB6 cell model and for tumor promotion in mouse skin carcinogenesis. In turn, activation of extracellular signal-regulated protein kinase (ERK) is required for AP-1 activation. We used three variants of the mouse epidermal cell line JB6 (Cl 30.7b, Cl 41, and Cl SC21) to identify events occurring on the pathway from ERK activation to AP-1 activation that may constitute molecular targets.

Mitogens such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and epidermal growth factor (EGF) activate the AP-1 transcription factor via the mitogen-activated protein kinase (MAPK) pathway. The MAPK family includes ERK, c-Jun N-terminal kinase/stress-activated kinases (JNK/SAPK), and p38 kinase. Only ERK is necessary for mitogen-induced AP-1 activation in JB6 cells. ERK 1 and ERK 2 are activated by mitogen stimulation through a cascade of kinases, including Ras, Raf, and MAPK kinase. Examination of mitogen stimulation of the MAPK pathway showed that the AP-1–nonresponsive, transformation-resistant (P^-) JB6 cell variant Cl 30.7b was deficient in ERK 1 and 2 proteins compared with AP-1–responsive, transformation-sensitive (P^+) cells (Figure 1). Restoration of

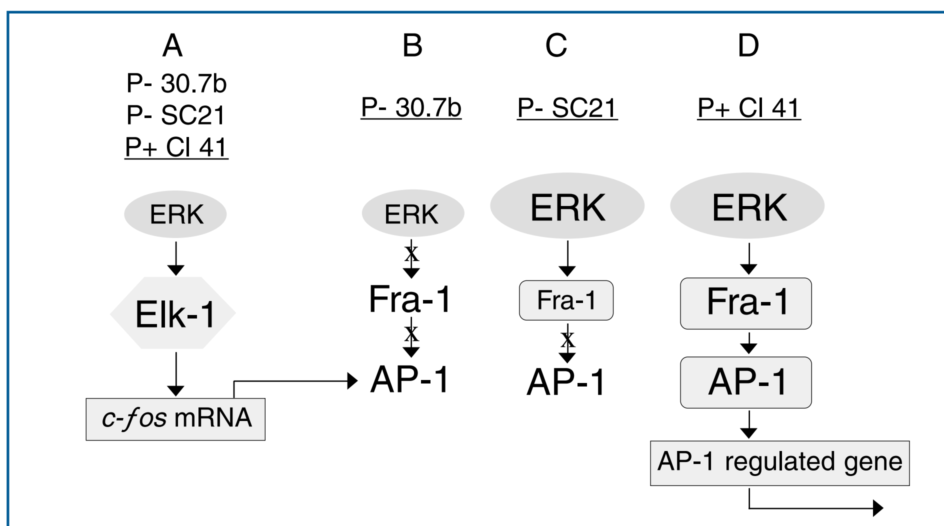

Figure 1. Pathway from extracellular signal-regulated protein kinase (ERK) to activator protein 1 (AP-1). (A) All three variants of the mouse JB6 epidermal cell line have sufficient ERK to drive activation of Elk-1 and c-*fos* expression. (B) JB6 Cl 30.7b cells do not have sufficient ERK to activate Fra-1, however, and thus the AP-1 promoter is not activated. (C) JB6 Cl SC21 cells resist mitogen-induced activation of AP-1 because although they have sufficient ERK to activate Fra-1, they lack sufficient endogenous Fra-1 to activate the AP-1 promoter. (D) JB6 Cl 41 cells have sufficient ERK and Fra-1 to complete the signal cascade from ERK to AP-1. Larger letters indicate sufficient protein; shaded areas indicate activated protein.

ERK levels by transfection of the P^- cells with an ERK 2 expression vector reconstituted TPA- or EGF-induced activation of AP-1 and transformation response in these cells. Conversely, inhibition of ERK activity in JB6 P^+ cells blocked TPA- and EGF-induced activation of AP-1 and transformation. Thus, activation of ERK 1, ERK 2, or both is required for mitogen activation of AP-1 and therefore for neoplastic transformation.

The MAPK cascade plays an important role in the control of cell proliferation and differentiation. Among the major nuclear targets of ERK are the ternary complex factor, Elk-1, and Sap-1a. Activation of the ternary complex factor activates transcription through an SRE site of immediate-early genes like c-*fos*, although c-*fos* transcription is not dependent solely on ERK. Little else is known of the ERK-dependent events required for AP-1 activation. Interestingly, c-*fos* transcription is not inhibited in the ERK-deficient P^- Cl 30.7b cells (Figure 1). To better understand the pathway(s) from mitogen-activated ERK to activation of AP-1 and neoplastic transformation, we used Gal4 fusions to compare the activation of AP-1 proteins in ERK-deficient P^- JB6 cells and ERK-sufficient P^+ JB6 cells.

AP-1 is a dimer of Jun and Fos proteins. The Fos family proteins, c-Fos and Fra-1, are activated in ERK-sufficient P^+ cells, but not in ERK-deficient P^- cells. TPA-induced activation can be inhibited by U0126, a small-molecule inhibitor of the MAPK kinase specific for activating ERK 1 and ERK 2, demonstrating that activation of both c-Fos and Fra-1 is dependent on ERK. P^- cells are not completely devoid of ERK 1 and ERK 2: Elk-1, a well-characterized ERK-dependent transcription factor, is activated by TPA even in ERK-deficient P^- cells. Thus, it appears that JB6 P^- Cl 30.7b cells have a level of ERK that is sufficient to activate Elk-1 but not to activate c-Fos or Fra-1 (Figure 1).

Interestingly, the DNA-bound, TPA-activated AP-1 complex from ERK-sufficient P^+ cells contains substantially more Fra-1 than that from ERK-deficient P^- cells. In contrast, the level of c-Fos in the TPA-treated AP-1 complex is slightly higher in P^- cells than in P^+ cells. Thus, the recruitment of Fra-1, and not c-Fos, into the active AP-1 complex appears to be pivotal for activation of AP-1–regulated transcription and tumor promotion.

Using Gal4-Fra1 fusions, we showed that the transactivation domain of Fra-1 can be activated by TPA in JB6 P^+ cells. This finding is noteworthy because earlier reports suggested that Fra-1 lacks a functional transactivation domain. It is also now clear that Fra-1 expression can activate transcription of putative AP-1–regulated genes and enhance transformation. We examined which sites in the transactivation domain of Fra-1 are required for its activation. MAPKs catalyze the phosphorylation of substrates containing a proline in the +1 site relative to the serine or threonine being phosphorylated. The potential ERK phosphorylation sites in the transactivation domain of the Gal4-Fra1 fusion (Ser 209, Thr 231, Thr 244, and Ser 269) were independently mutated to Ala by site-directed mutagenesis. Of these potential ERK phosphorylation sites, only the Thr 231-to-Ala mutation resulted in a loss of TPA-induced activation of Fra-1. These results support the hypothesis that the transactivation domain of Fra-1 is activated by ERK-dependent phosphorylation of Thr 231. Fra-1 appears to be required for activation of AP-1 in JB6 P^+ cells, as Cl 41 cells transiently transfected with antisense *fra-1* cDNA were blocked for both basal and TPA-induced activation of AP-1 in a dose-dependent manner.

While the transformation resistance of the P^- 30.7b clone is due to an insufficient amount of ERK 1 and ERK 2 needed to phosphorylate Fra-1, another P^- clone, SC21, is not ERK deficient (Figure 1). The resistance of SC21 cells to AP-1 activation and transformation is attributable to an insufficient level of Fra-1 protein needed to complete the signaling from activated ERK to activation of AP-1. Expression of Fra-1 in SC21 cells following transfection with a cytomegalovirus (CMV) promoter-driven *fra-1* expression vector restored the MAPK cascade leading to TPA-induced activation of AP-1. These results suggest that synthesis of

Fra-1 (and not Fra-1 proteolysis) is the limiting factor rendering the SC21 cells resistant to AP-1 induction.

Overexpression of Fra-1 can activate transcription of putative AP-1–regulated genes and enhance tumorigenesis. Indeed, in JB6 P^+ Cl 41 cells, overexpression of *fra-1* cDNA significantly enhances AP-1–regulated transcription. To determine if Thr 231 is required for activation of AP-1 in JB6 cells, Thr 231 in the *fra-1* cDNA was mutated to Ala. Expression of T231A-mutated Fra-1 does not activate AP-1, indicating that phosphorylation of Thr 231 is necessary for activation of AP-1. The CMV-*fra-1-T231A* does not function as a dominant-negative mutant.

Understanding the AP-1–dependent mechanisms by which chronic exposure of initiated cells to tumor promoters leads to tumorigenesis may allow us to prevent or reverse tumor promotion. We have contributed to this understanding by demonstrating that in the mouse epidermal JB6 model, Fra-1 appears necessary to complete the signaling cascade leading to AP-1 activation and that a critical threshold level of ERK 1, ERK 2, or both is needed to activate Fra-1. Discovery of Fra-1–dependent target genes may reveal important new molecular targets for cancer prevention.

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Dissecting the Many Roles of XIAP

Reffey SB, Wurthner J, Parks WT, Roberts AB, and Duckett CS. X-linked inhibitor of apoptosis protein functions as a co-factor in transforming growth factor-ß signaling. *J Biol Chem* 276: 26542-9, 2001.

Apoptosis is an evolutionarily conserved, non-inflammatory process that plays a critical role during development and tissue homeostasis. This highly regulated process of cell death also serves to remove damaged or extraneous cells from an organism. Disruption of the apoptotic pathway occurs in a wide variety of diseases, including cancer, AIDS, other autoimmune diseases, and neurodegeneration. Apoptotic cells undergo a regulated autodigestion that involves disruption of cytoskeletal integrity, cell shrinkage, nuclear condensation, and endonuclease activation. The chief effectors of this pathway are a family of cysteine-aspartate proteases called the caspases. Caspase activity is regulated by several families of both pro- and anti-apoptotic proteins, including the inhibitor of apoptosis protein (IAP) family. IAPs suppress apoptotic cell death by directly binding to and inactivating the enzymatic activity of the caspases.

One member of this family, the X-linked inhibitor of apoptosis protein (XIAP), has been shown to bind to and inhibit both upstream initiator caspases (caspase-9) and downstream effector caspases (caspase-3 and -7). Recent data have indicated that XIAP—and IAPs in general—play other significant roles within the cell as well. For example, XIAP has been implicated in the regulation of c-Jun N-terminal kinase (JNK), a stress-induced kinase. Similarly, XIAP has been identified as an activator of nuclear factor kappa B (NF-κB), a pleiotropic transcription factor that regulates expression of a wide range of acute-phase and immediate-early genes.

Several other members of the IAP family are involved in signaling cascades. For example, the *Drosophila* IAPs, dIAP-1 and dIAP-2/dILP, have been shown to interact with thickveins. Thickveins is a type I serine-threonine kinase receptor homologous to the bone morphogenetic protein type I receptor, a member of the transforming growth factor beta (TGF-ß) receptor superfamily.

The TGF-ß superfamily encodes a group of related ligands that includes TGF-ß, bone morphogenetic protein, Mullerian inhibitory substance, and the activins/inhibins. Depending on the cellular context, these ligands elicit an array of cellular responses, including apoptosis, cell cycle arrest, and differentiation. Signal transduction initiated by these cytokines involves two classes of single-pass transmembrane receptor serine-threonine kinases. Ligand binding to type II receptors leads to recruitment and transphosphorylation of type I receptors, which in turn associate with and phosphorylate a subset of the Smad family of signal transduction intermediates. Upon phosphorylation the activated Smads dissociate from the receptor; hetero-oligomerize with a common partner, Smad4; and translocate to the nucleus, where they take part in transcriptional activation of target genes.

Interestingly, both TGF-ß and XIAP can specifically activate JNK. Furthermore, Smad proteins have been shown to cooperate with the activator protein 1 transcription factor, which is activated by JNK. Because of this tight link between the TGF-ß and JNK signaling pathways, we became interested in a possible role for XIAP in TGF-ß–mediated signal transduction. Co-precipitation experiments between XIAP and a panel of TGF-ß type I receptors as well as immunofluorescent co-localization studies revealed that XIAP can associate with several of the TGF-ß type I receptors, including TGF-ß RI, the receptor responsible for activating the TGF-ß–specific signaling cascade. This association was highly specific for XIAP, as other

XIAP can be placed in a central location for coordinating signaling from the TGF-ß type I receptor and for activating both JNK and NF-κB . . . . In addition, XIAP is a multifunctional protein involved in not only TGF-ß–mediated signal transduction, but also inhibition of caspase activity.

mammalian IAP proteins (c-IAP1 and c-IAP2) did not co-precipitate with this receptor.

Since an interaction was seen between XIAP and the TGF-ß type I receptor, we next examined the role of XIAP in TGF-ß—mediated gene activation. Reporter assays revealed that XIAP significantly enhanced TGF-ß—mediated activation of a TGF-ß—responsive promoter and that transient overexpression of XIAP alone activated this promoter. This activation required the C-terminus of XIAP, which contains a RING finger known to be involved in ubiquitin conjugation. We also found that Smad4, the common signaling intermediate of the TGF-ß superfamily, was required for activation of the same TGF-ß—responsive promoter as well as for activation of both JNK and NF-κB by XIAP, although these proteins (XIAP and Smad4) do not directly associate with one another. Interestingly, the C-terminal RING domain of XIAP was required for activation of NF-κB and the TGF-ß—responsive promoter but was not involved in the activation of JNK, suggesting that these functions can be separated.

Because XIAP is known to be a caspase inhibitor, we wanted to know if this function is dependent on Smad4. Using a substrate of caspase-3 to directly measure caspase enzymatic activity, we found that a dominant-negative Smad4 mutant did not affect the ability of XIAP to inhibit caspase activity. This observation suggests that the anti-apoptotic functions of XIAP are independent of its roles in the activation of JNK, NF-κB, and TGF-ß—mediated signaling. Thus, XIAP can be placed in a central location for coordinating signaling from the TGF-ß type I receptor and for activating both JNK and NF-κB, co-factors involved in transcribing subsets of TGF-ß—responsive genes. In addition, XIAP is a multifunctional protein involved in not only TGF-ß—mediated signal transduction, but also inhibition of caspase activity. These two functions are distinct in that the signaling properties of XIAP require Smad4, while the anti-apoptotic functions are Smad4 independent.

Reconciling these two functions of XIAP may lead to a greater understanding of cancer progression. As tumors progress, they frequently become refractory to TGF-ß—mediated growth arrest and to various apoptotic signals. Since XIAP plays a role in both pathways, this protein may help determine cell fate and thus may be an important molecular target.

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■ BIOTECHNOLOGY RESOURCES

After Genomics Comes the Hard Part: Converting Genes to Proteins

Compared with DNA and RNA, proteins are whimsical, erratic, unpredictable beasts: so many different amino acids that interact with each other in so many ways to form helices, bends, barrels, sheets, and fingers. Scientific factories can crank out millions of bases of sequence every day, but translating one gene into its soluble, active, native protein can take a very great deal of time and effort.

The NCI/SAIC Protein Expression Laboratory (PEL) in Frederick can help NCI investigators get the proteins they need to move their research forward. As part of the Research Technology Program (http://web.ncifcrf.gov/rtp/), which was established by the Office of the Director to provide state-of-the-art technology to NCI investigators, the PEL is directed by Jim Hartley, Ph.D. Dr. Hartley—who arrived in November 2001 after 20 years at a Rockville company known successively as Bethesda Research Laboratories, Life Technologies, and Invitrogen—constructed the original "ladder" DNA standards, invented PCR methods, and helped invent and develop the Gateway™ recombinational cloning technology.

The PEL is comprised of five groups, which can work independently or together to assist investigators in their research programs:

- The **Cloning and Expression Optimization** group, headed by Dominic Esposito, Ph.D., constructs expression clones and optimizes expression in *Escherichia coli* if required. The process often uses PCR and the Gateway™ *in vitro* recombination system, so that multiple expression hosts and contexts can be tested in parallel. More than 50 expression vectors for *E. coli*, yeast, mammalian, and insect cells are available, and investigators' own vectors are welcome.

- The **Microbial Fermentation** group, headed by David Miller, can grow *E. coli* and other microbes—in shake flasks or multiple fermentation tanks—with working volumes of up to 60 liters. Equipment is computer controlled and monitored, allowing, for example, fed batch growth to OD's above 200 for *E. coli*. Shake flasks typically reach OD's of 5–10.

- The **Eukaryotic Cell Production** group, headed by Ralph (Butch) Hopkins, can maintain or amplify investigators' cell lines; produce, harvest, concentrate, and assay conditioned media containing antibodies or other proteins of interest; and transfect DNA for the transient or stable expression of recombinant proteins. Amplification, titering, and optimization of baculovirus infections are standard procedures, and scale-up to many liters is routinely performed.

- The **Protein Purification** group, headed by William Gillette, Ph.D., uses computer-controlled chromatography stations to achieve rapid, reproducible, scaleable protein purifications. Purifications can follow established protocols or be developed on a case-by-case basis. Polyhistidine fusions are often used because of their small size and compatibility with structural studies.

- The ***In Vitro* Transcription/ Translation** group, headed by Deb Chatterjee, Ph.D., produces proteins from plasmids or PCR products, using *E. coli* cell extracts. Dr. Chatterjee, who has wide experience in gene expression and protein engineering as well as *in vitro* transcription, joined the PEL in July 2002. By removing the "*in vivo*" part of the process, toxicity considerations are eliminated and much higher throughputs are possible.

Examples of recent PEL projects include:

- Greatly increasing the solubility of a human protein by expressing it with a maltose binding protein fusion tag (Figure 1).

- Fusing three different genes to four different epitope and fluorescent tags for expression studies in mammalian cells.

- Fermenting and purifying protein labeled with ^{15}N for nuclear magnetic resonance studies.

- Producing a small protein with bizarre amino acid composition by generating inclusion bodies in *E. coli*.

- Significantly increasing the expression of a mammalian gene by using an *E. coli* strain that supplies rare tRNAs.

- Cloning, transfecting, and expressing a human viral protein in insect cells.

To learn more about the resources available at the PEL, visit the PEL Web site at http://web.ncifcrf.gov/rtp/PEL.asp, or contact Dr. Hartley.

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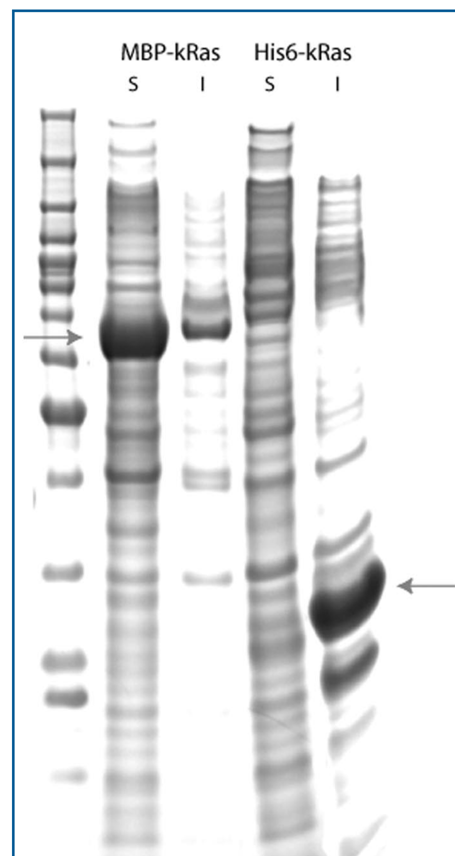

Figure 1. The choice of fusion tags can alter the solubility of a human protein expressed in *E. coli*. The K-*ras* gene was cloned into a Gateway™ vector, then subcloned into N-terminal MBP (maltose binding protein) or N-terminal His6 fusion vectors. Expression was induced with IPTG. S, soluble; I, insoluble. Arrows show the predicted sizes of the two fusion proteins.

Stefan Ambs, Ph.D.

Born in Germany, Stefan Ambs, Ph.D., received his Master's degree in biochemistry from the University of Tübingen and completed his Ph.D. thesis at the Institute of Toxicology, University of Würzburg, Germany. He came to the United States in 1992, as a postdoctoral fellow in the Laboratory of Human Carcinogenesis (LHC), NCI. Dr. Ambs then continued his research at a biotechnology company, Megabios Corporation, in Burlingame, CA, and at the Aventis Genomics Center in Cambridge, MA. He recently returned to the LHC as a tenure-track investigator in molecular epidemiology.

A biochemist by training, Dr. Ambs has spent many years exploring the role of nitric oxide in the biology of human cancer. His research has revealed that, at moderate concentrations, nitric oxide increases tumor vascularization and promotes growth of *p53*-mutant cells. His research has established a model of nitric oxide-induced cancer progression.

In his new position at the LHC, Dr. Ambs will continue to follow his interest in the relationship between pro-inflammatory molecules (nitric oxide, cytokines, eicosanoids), angiogenesis, and cancer progression. He will explore inter-individual variations in cancer risk defined by allele variants of genes, environmental risk factors, and endogenous risk factors. His laboratory is currently testing the hypothesis that allele variants of genes in the inflammation, hormone, and angiogenesis pathways modulate the risk for human breast and prostate cancers. His research goals are to identify genetic and environmental risk factors and to explore the functional aspects of allele variants in laboratory models. The program of Dr. Ambs' laboratory will therefore investigate both the aspects of cancer susceptibility in a population, based on association, and the underlying molecular mechanisms that cause the susceptibility.

Dr. Ambs' research will combine traditional epidemiology with laboratory investigations. Already Dr. Ambs has established a case-control study of breast cancer in Maryland and has formed a collaboration to investigate prostate cancer in a Danish case-control study. Future projects are aimed at identifying risk factors for inflammatory breast cancer and for esophageal cancer as the condition relates to Barrett's syndrome.

Dr. Ambs

Dr. Ambs is married and lives in Silver Spring, MD. In his spare time, he enjoys fishing and exploring native orchid species in North America.

Administrative Links

Hold on Title 42 Appointments and Pay Adjustments for Salaries over $100,000

In an effort to create a standard system and criteria for salary setting within the Title 42 authority across NIH, Dr. Elias Zerhouni has issued a temporary freeze on Title 42 appointments and pay adjustments for salaries greater than $100,000. Dr. Ruth Kirschstein will lead a group of senior OD and IC staff with the charge to make recommendations that should allow delegation of authority to IC leadership.

Travel Policy Changes and Clarifications

Travelers will now be allowed to use annual leave in conjunction with official foreign travel not to exceed one (1) day of leave for every day of official duty travel. (Previous Department policy restricted annual leave in association with foreign travel.) NIH employees may not receive per diem for travel within the local area. Exceptions may be granted by an ARC Manager **only** if the following conditions exist:

1) Travel location is more than 35 miles from official duty station **and** residence, and
2) Employee is required to be in official duty status for at least the normal work week, or
3) Employee must stay at the travel location beyond a reasonable hour.

Job Applicants Need Facts on Standards of Ethical Conduct

Federal supervisors should ensure that job applicants—particularly individuals applying for senior positions—are fully aware of conflict of interest and financial disclosure requirements prior to receiving an employment offer. More information on avoiding conflicts of interest and standards of ethical conduct can be found in the NIH Policy Manual at http://www1.od.nih.gov/oma/manualchapters/person/2300-735-1/. There is also an NIH Fact Sheet at http://ethics.od.nih.gov/FactSheet.htm. You may also contact the NCI Ethics Office at 301-496-1148.

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