Children’s Hospital of Philadelphia

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2012 – December 31, 2012

Formula Grant Overview

The Children’s Hospital of Philadelphia received $3,640,981 in formula funds for the grant award period January 1, 2009 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Role of Crk and CrkL in Normal and Neoplastic Growth - Normal growth requires a delicate balance among the processes that grow new cells and those that eliminate superfluous cells. Cancer arises because of a breakdown in this balance between cell division and cell death. Cancer cells continue to grow as they fail to respond to signals that tell them to stop. The investigator proposes to study the role of two growth control genes, Crk and Crk-like (CrkL), in normal growth and development as well as in cancer cells. The strategy involves the use of genetic techniques to remove Crk and CrkL from different kinds of cells, in culture and in mice, and then to examine the consequences for cell growth. In addition, the genes will be removed from tumor cells to determine if they function in similar or different ways in cancer.

Duration of Project

1/1/2009 - 12/31/2012

Project Overview

Crk and CrkL are adaptor proteins that function in signal transduction processes during normal and neoplastic growth. Understanding the specific biological functions of Crk and CrkL has been challenging because deletion of either gene from the mouse germline results in embryonic or early postnatal lethality, and because both proteins function very similarly in biochemical and molecular assays. Previously, the Curran laboratory generated mutant mice carrying floxed alleles of Crk and CrkL allowing conditional mutation using the CRE system. Ablation of both Crk and CrkL in neuronal precursor cells demonstrated that they provide specific overlapping functions downstream of Reelin in the control of radial neuronal migration during brain development. The present project represents an expansion and new direction of this study to investigate the role of Crk and CrkL in a range of cell types in vitro and in vivo. Establishment of the \(Crk^{fl/fl}/CrkL^{fl/fl}\) strain of mice as well as \(Crk^{fl/fl}\) and \(CrkL^{fl/fl}\) strains provides a unique and powerful experimental tool that enables the studies proposed in specific aims 1-3. The overall hypothesis to be addressed is that Crk and CrkL function in signal transduction processes by
linking tyrosine phosphorylation signals to alterations in cell growth, adhesion and migration properties. Three specific aims will be addressed that focus on the role of Crk and CrkL in fibroblast growth (Aim 1), in other cell types in vitro and in vivo (Aim 2) and in cell transformation and tumor growth (Aim 3). Comparison of phenotypes obtained from \( \text{Crk}^{\text{fl/fl}}/\text{CrkL}^{\text{fl/fl}} \) cells and tissues with those from \( \text{Crk}^{\text{fl/fl}} \) and \( \text{CrkL}^{\text{fl/fl}} \) mice will provide detailed understanding of how Crk and CrkL play both overlapping and individually unique functions in various cells and tissues. The initial study of Crk and CrkL functions in fibroblast cells will elucidate their general role in cell biological processes. Understanding both conserved and specific functions of Crk and CrkL in various cell types will enable us to address fundamental questions in birth defects and cancer.

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**Expected Research Outcomes and Benefits**

This research will reveal the basic mechanisms whereby certain oncogenes (\( \text{Crk} \) and \( \text{CrkL} \)) affect the growth of normal cells as well as tumor cells. Insights from the basic observations concerning the growth of normal cells lacking Crk and CrkL will provide clues about the way in which cancer cells grow abnormally. Studies in mice will reveal the biological functions of Crk and CrkL in different tissues. Deleting Crk and CrkL in cancer cells will reveal whether these proteins are required for the formation and continued growth of tumors. This information will be used to predict new approaches for intervention in the treatment of cancer.

**Summary of Research Completed**

*Individual contribution of Crk and CrkL to cytoskeletal integrity*

To investigate the individual contributions of CrkI, CrkII and CrkL to the maintenance of cell shape, the researchers used the Nikon NIS element program for quantitative analysis of cytoplasmic area (in square micrometer) and other parameters as reported in the previous year. Crk/CrkL double floxed cells were coinfected with lentiviral vectors expressing GFP/Cre together with vectors expressing various Crk and CrkL proteins. At 5 days post infection (DPI), images of fluorescent cells were captured and the cytoplasmic areas were determined as the researchers reported in the previous year. Expression of exogenous CrkI or CrkL caused a
modest but significant inhibition of the morphological alteration caused by loss of both Crk and CrkL (increases of cytoplasmic areas to 559 ± 82 by CrkI expression and to 524 ± 58 by CrkL expression from 355 ± 34). While some infected cells still showed the typical morphological conversion, others retained a larger surface area. CrkII was more effective than CrkI or CrkL in preventing morphological alteration (an increase of cytoplasmic areas to 1173 ± 195 by CrkII expression). Analyses of other cell size parameters, such as equivalent diameter (diameter of a circle with the same area as the measured object, i.e. square root (4*area/π)) or perimeter, gave results comparable with the cytoplasmic area findings. Equivalent diameters (in micrometer) increased to 25.2 ± 1.8 by CrkI, 24.7 ± 1.4 by CrkL, and 36.4 ± 3.3 by CrkII from 20.5 ± 0.9. Perimeters (in micrometer) increased to 113.7 ± 9.8 by CrkI, 93.0 ± 6.7 by CrkL, and 193.4 ± 21.4 by CrkII from 70.5 ± 3.5. These data suggest that CrkI, CrkII and CrkL all contribute to cytoskeletal connection with the extracellular matrix, although CrkII seems to play a dominant role. Mutants of Crk and CrkL containing only SH2 domains failed to rescue morphological conversion. Cytoplasmic areas for cells with CrkSH2 and CrkLSH2 expressions were 323 ± 54 and 466 ± 68, respectively, which were not significantly different from 355 ± 34 by the mCherry vector expression. Furthermore, the SH2 only mutants promoted significant morphological alterations when expressed by themselves, even in the presence of endogenous Crk and CrkL. The cytoplasmic area decreased to 530 ± 68 by CrkSH2 and to 825 ± 99 by CrkLSH2 from 1335 ± 130. These findings suggest that Crk and CrkL mediate the connection of the cytoskeleton with the extracellular matrix through both SH2 and SH3 domains, with SH2-only mutants acting as dominant-interfering molecules.

To confirm the individual contributions of Crk family proteins, the researchers infected single floxed cells with a lentiviral vector expressing GFP and Cre to see whether deletion of either Crk or CrkL alone caused any morphological alteration. Deletion of either Crk or CrkL did not cause the drastic morphological changes seen in cells lacking both Crk and CrkL. However, close examination revealed that some GFP-positive cells lacking either Crk or CrkL showed modest morphological effects. There was a modest but significant decrease in cytoplasmic area in cells lacking either Crk or CrkL (68.0 ± 8.4% and 73.9 ± 5.7% of the normal size, respectively). The reduction in cell size that occurred after ablation of both Crk and CrkL (66.5 ± 2.0%) was greater than the combination of the effects caused by individual ablation of Crk and CrkL (49.7% based on 1 - 0.680 x 0.739 = 0.497). Analyses of equivalent diameter and perimeter also showed similar reductions in the absence of either Crk or CrkL and greater reductions in the absence of both Crk and CrkL. Equivalent diameters decreased from 39.2 ± 1.7 to 32.0 ± 1.8 in the absence of Crk, from 49.0 ± 2.1 to 42.6 ± 1.6 in the absence of CrkL, and from 39.9 ± 1.3 to 24.2 ± 0.7 in the absence of both Crk and CrkL. Perimeters decreased from 180.5 ± 9.6 to 138.9 ± 9.5 in the absence of Crk, from 237.9 ± 12.2 to 192.1 ± 8.4 in the absence of CrkL, and from 179.1 ± 7.0 to 95.0 ± 3.7 in the absence of both Crk and CrkL. The greater reduction in the cell size in the absence of both Crk and CrkL is consistent with the drastic morphological alterations observed in cells lacking both Crk and CrkL. These findings suggest that CrkI, CrkII and CrkL play essential, overlapping roles in the maintenance of cell structure. Taken together, the results indicate that CrkI, CrkII, and CrkL are all required for mediating communication between the intracellular environment and the extracellular matrix.
Requirement of Crk and CrkL in cell transformation

Previously, the researchers demonstrated that in the absence of Crk or CrkL cells can’t undergo full transformation process. The researchers showed that Crk or CrkL knockout cells present less obvious transformation phenotype and form fewer colonies in soft agar assay after transformation induced with fos or ras oncogenes. Additionally researchers had indications that Dnmt1 methyltransferase may be involved in the effect of v-fos induced transformation in Crk-/– or CrkL-/– cells. However further studies did not confirm the role of Dnmt1 in the absence Crk or CrkL. Additionally the researchers tested whether removal of Crk or/and CrkL can disrupt the established transformation. Fibroblasts derived from single floxed Crk (Crk f/f) or CrkL (CrkL f/f) mice or Crk/CrkL double floxed mice were used for the experiments. Fibroblast cells were cultured and infected with a retroviral vector expressing v-fos or h-ras-V12 to induce transformation and at 48 h after initiation of transformation cells were infected with a lentiviral vector expressing Cre plus GFP and observed for morphological changes. Five days after the initial infection researchers observed morphological changes in WT cells transformed with v-fos or h-ras-V12. At the same time it was found that removal of CrkL noticeably reduced the transformation phenotype of v-fos or h-ras-V12 while compared to wild type cells. Moreover, independent removal of Crk also visibly diminished the transformation phenotype in cells undergoing v-fos or h-ras-V12 induced alterations. Removal of both Crk and CrkL at the same time from the cells undergoing transformation caused a reduction in the number of cells. However, this result can’t be interpreted since removal of both Crk and CrkL from the WT cells alters the morphology of the cells, making them smaller, round and retractile. The new results indicate that Crk and CrkL play essential roles in maintaining the transformation phenotype. Taken together researchers presented evidence that Crk or CrkL expression is necessary during the initiation and early stage of transformation.

Generation and characterization of T cell-specific Crk/CrkL deficient mice.

c-Abl family kinases are required for T cell actin responses and T cell migration. Abl family kinases are known to phosphorylate Y221 in CrkII and Y207 in CrkL, but little is known about the identity of Abl kinase substrates in T cells in vivo. To study the in vivo function of Crk and CrkL in T cells, the researchers in collaboration with other researchers at the same institute generated mice that are conditionally deficient for Crk and CrkL in the mature T cell compartment by breeding mice bearing loxP-flanked Crk and CrkL alleles with mice carrying the Cre recombinase gene under the transcriptional control of the endogenous CD4 promoter. T cell-specific Crk/CrkL knockout mice were born in expected mendelian ratios, and the spleen, thymus and lymph nodes exhibited normal cellularity. In addition, T cell developed normally.

Crk/CrkL-deficient T cells show defects in Rap1 activation, adhesion and migration.

To test whether Crk and CrkL are required for activation of Rap1 and Rac1 in chemokine signaling pathways, the researchers stimulated CD4+ T cell blasts with CCL21, the ligand for CCR7 that regulates T cell trafficking in vivo. Normal T cells exhibited Rac1 activation, and neither the magnitude nor the kinetics of Rac1 activation was affected by deficiency of Crk and CrkL. However, Rap1 activation was attenuated in Crk/CrkL deficient T cells, demonstrating that upon chemokine stimulation Rap1 activation depends on Crk and CrkL. Since one of the important biological effects of Rap1 signaling is the acute regulation of cell adhesion mediated by the activation of integrins, the researchers tested if the impaired Rap1 activation affects T cell adhesion in response to chemokine stimulation. Anti-CD3 treatment increased T cell adhesion to
ICAM-1, the ligand that binds to the β2 integrin LFA-1 by 50%. However, Crk/CrkL-deficient T cells showed 2-fold reduction in adhesion to ICAM-1 under both unstimulated and anti-CD3 stimulated conditions. This phenotype was even more dramatic when T cells were activated by chemokines such as CXCL12 and CCL21. The results show that Crk and CrkL are required for integrin-dependent T cell adhesion. The researchers also asked whether Crk and CrkL are functionally important for T cell migration. CCL21, a CCR7 ligand that regulates T cell trafficking to lymphoid organs in vivo, and CXCL10, a CXCR3 ligand that preferentially regulates Th1 cell trafficking, induced wild-type T cell migration in a dose-dependent manner. In contrast, Crk/CrkL-deficient T cells displayed 20-50% reductions in migration in response to both chemokines. Taken together, these results demonstrate that Crk and CrkL are required for adhesion and migration of T cells.

Research Project 2: Project Title and Purpose

*Gene Transfer of Activated FVII for the Treatment of Inherited Coagulation and Platelet Disorders* - The treatment of hemophilia (a bleeding disorder) is complicated by the development of antibodies (inhibitors) against the therapeutic factor (Factor VIII or Factor IX) infused in response to bleeds. Clinical data demonstrate the successful but costly treatment of such patients with infusion of high-dose FVIIa. To address this with gene transfer, an engineered FVIIa transgene has been developed and delivered, the expression of which resulted in a substantial improvement in the bleeding diathesis of hemophilic mice and dogs. The experiments here aim to further demonstrate the feasibility of this approach in animals with inhibitors as well as other inherited coagulation defects, and the further optimization of the gene delivery vehicle and transgene. These are critical steps towards a clinical application that can result in a single, simplified treatment for bleeding disorders.

Duration of Project

1/1/2009 - 12/31/2012

Project Overview

A major complication in the treatment of hemophilia (Factor VIII [FVIII] or Factor IX [FIX] deficiency) using protein replacement is the development of neutralizing antibodies against the infused factor (FVIII or FIX) that occurs in a considerable number of severe hemophilia cases. Over the past 15 years, such patients, as well as those with congenital FVII deficiency or inherited platelet disorders, have been treated successfully with recombinant human Factor VIIa (rhFVIIa). Based on the current understanding of coagulation, rhFVIIa can simplify patient management to a single product, but the short half-life, multiple dosing and high cost have prevented such an approach. Gene transfer has been proposed as an alternative to protein infusion in the management of bleeding in hemophilia. Specifically, gene transfer with FVIIa can potentially bypass the shortcomings of rhFVIIa protein therapy by conferring long-term expression and offer an immunological (since all hemophilia patients are tolerant to FVIIa) as well as a therapeutic advantage. The researcher has previously engineered a FVII transgene that is secreted in its active form (FVIIa) and demonstrated improvement of the bleeding diathesis in hemophilic mice and dogs, by viral-vector mediated, liver-directed, FVIIa gene transfer.
Moreover, the researcher’s data from hemophilic dogs suggest that efficacy is proportional to the vector dose utilized and thus disorders that are currently treated with low-dose rhFVIIa (e.g., FVII deficiency) will be appealing targets for such gene transfer. However, further development of this therapeutic relies on aspects that are currently unknown and thus the researcher is pursuing experiments to address them, as follows: (1) What is the efficacy of this gene transfer approach in hemophilia with inhibitors or platelet disorders? For this, the researcher will use hemophilic mouse models to generate inhibitors against FVIII or FIX, or use mouse models of platelet disorders and subsequently deliver mouse FVIIa via gene transfer in order to study efficacy in vitro and in vivo. (2) Can the vector be optimized to allow for easier scalability and avoiding adverse events from high viral loads? For this, the researcher will perform an optimization of vector and transgene and compare efficacy in mice and hemophilic dogs. (3) Can this approach be utilized in congenital FVII deficiency? For this, the researcher will use a newly established dog model of FVII deficiency to evaluate the effects on bleeding diathesis following cFVIIa gene transfer. These answers will greatly impact this gene-based approach that can potentially provide a unified, simpler alternative to hemophilia treatment.

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**Expected Research Outcomes and Benefits**

A major complication in the treatment of hemophilia (a bleeding disorder) with protein infusion is the development of antibodies against the infused factor (Factor VIII [FVIII] or Factor IX [FIX]) occurring in a considerable number of severe cases of the disease. Infusion of high doses of a purified factor (activated FVII [FVIIa], that these patients already have), has demonstrated excellent efficacy in such patients but its short circulating life in blood results in a substantial financial burden. Alternatively, gene therapy may offer long-term correction of the bleeding diathesis via stable expression of the appropriate gene(s). The researcher has developed such an approach with a gene coding for FVIIa and has shown correction of the bleeding diathesis in both hemophilic mice and dogs. However, certain key questions of using this strategy remain unanswered. Specifically, the first aim is to investigate whether FVIIa gene transfer can correct the bleeding phenotype in a mouse model of hemophilia in the presence of inhibitory antibodies to FVIII or FIX or in mice with platelet disorder, as has been shown with FVIIa infusion. These animal models will provide the experimental confirmation for the researcher’s proposed gene transfer approach. Since high doses were necessary to correct the bleeding diathesis in hemophilic dogs, the researcher also wants to improve the vector/transgene that can lower the
effective dose. This is important since high vector doses impose scalability issues for human application and may have adverse events resulting from high viral loads. The last aim is to investigate whether gene transfer with FVIIa can correct the bleeding diathesis in a newly described dog model of FVII deficiency. The researcher’s data in hemophilic dogs suggest that efficacy is proportional to the infused vector dose for gene transfer; since FVII deficiency is currently treated with low-dose FVIIa protein infusion, it would thus be an ideal and feasible target for FVIIa gene transfer. Overall, the experiments aim to further preclinical development of this approach that may lead to a single, simplified treatment for bleeding disorders.

Summary of Research Completed

Experiments in Aim 1 were accomplished in the previous reporting period. In this reporting period (7/1/2012 – 12/31/2012) the researcher’s lab performed experiments as described in Aim 2-3, according to the milestones set in the original project description. These experiments and the obtained results are described in detail below:

AIM 2

Optimized vectors for FVIIa gene transfer

Previously, the researcher constructed an AAV vector backbone that upon AAV packaging will result in an AAV vector with a double-stranded genome expressing a codon-optimized canine Factor VIIa (cFVIIa-CO). The vector was named scAAV-cFVIIa-CO and showed a ten-fold vector dose advantage compared to a single-stranded AAV vector expression canine FVIIa (no-codon-optimized). This advantage was determined using a prothrombin time clotting assay. The most appropriate model to test the efficacy of the scAAV-cFVIIa-CO versus the single stranded vector is transduction of hemophilic mice. The researcher initially generated a batch of scAAV-cFVIIa-CO that had a very low yield, compared to a single-stranded AAV vector expressing canine FVIIa. This low-yield preparation was utilized in its entirety for all the experiments previously described in hemostatically normal mice (in the previous reporting period). As a result, in this reporting period, the researcher initiated a new vector preparation for scAAV-cFVIIa-CO but has not yet evaluated its performance in hemophilic mice.

Milestones:  
(1) Test the improved vector preparations (expressing cFVIIa) for their hemostatic effects in HA mice (Aim 2): partially accomplished (initiated new vector preparation)  
(2) Administer the improved vectors (expressing cFVIIa) in hemophilia A or B dogs, depending on the availability (Dr. Timothy C. Nichols, UNC Chapel Hill colony). Begin monitoring of hemostatic efficacy in infused dogs (Aim 2): not accomplished.  
(3) Monitor the hemostatic efficacy in infused HA or HB dogs with the improved vectors expressing cFVIIa. Perform in vitro assays to determine safety on samples taken at various timepoints. Not accomplished
**AIM 3**

**Expression of cFVII or cFVIIa in dogs with FVII deficiency**

Previously, the researcher established a clotting based assay to detect canine FVII (cFVII) activity in dog plasma. This was done to quantify the expression of cFVII in FVII deficient dog(s) that receive an AAV serotype 8 vector expressing cFVII (AAV-cFVII). Moreover, the researcher showed initial data on a FVII deficient dog that received a dose of 4.95E13 vector genomes/kg.

Here, the researcher developed an additional in vitro assay to determine cFVII activity in canine FVII deficient plasma. Rotational thromboelastometry (ROTEM) can provide clot dynamics information as a function of time. In this assay, the sample is activated (tissue factor [TF] or contact pathway), and clot formation results in a time-dependent change in clot elasticity. The instrument measures this elasticity via an oscillating pin and depicts it as clot amplitude (positive/negative, Figure 1). A typical thromboelastogram is shown in Figure 1B. Here, the researcher used a plasma adaptation of this assay to establish parameters that can be used to monitor cFVII levels in FVII deficient dogs. Using pooled mixtures of canine normal and FVII deficient plasma to obtain defined cFVII activity levels, the researcher monitored clot formation vs. time using TF as initiator. Evaluation of all parameters from the derived clotting profiles, demonstrated that clot time (CT) and time to reach maximum clot velocity (MAXV-t) correlated with FVII activity. An explanation of these parameters is shown in Figure 1B. The experimental data are shown in Figure 2. As FVII activity increases, the time to reach MAXV (MAXV-t) as well as the clot time (CT) decreases. These data establish an additional assay that can monitor a wide range of cFVII activity that will also be utilized for the AAV-treated FVII deficient dogs.

The researcher also assayed additional timepoint samples from the FVII deficient dog infused with AAV-cFVII that extend beyond what was shown in the previous report. A prothrombin time assay was used to follow cFVII expression as well as ROTEM analysis. Data are shown in Figure 3 and demonstrate stable cFVII expression as evidenced by prothrombin clotting times (Figure 3A) and CT and MAXV-t (Figure 3B). The experimental points also suggest that the vector dose administered to this FVII deficient dog resulted in cFVII expression at or better than normal.

**Milestones:**

1. Test the vector preparations (expressing cFVII or cFVIIa) for their hemostatic effects in HA or HB mice: not accomplished (utilized cFVII deficient dogs instead)
2. Monitor the hemostatic efficacy in infused FVII deficient dogs with the vectors expressing cFVIIa or cFVII zymogen; perform in vitro assays to determine safety on samples taken at various timepoints: Partially accomplished (monitored efficacy)
Figure 1. (A) In a rotational thromboelastrometry instrument the sample is placed in a cup and following activation, a pin oscillates clockwise/anticlockwise. As the clot develops, its elasticity changes and is translated as clot amplitude, for each direction of movement (positive/negative). (B) In the resulting thromboelastogram (combined clot amplitudes), the clot time (CT) is the time to initial fibrin formation (Left). The first derivative of the clot amplitude (velocity) can be obtained (right) and the peak is called maximum velocity (MAXV). The time to reach MAXV is called MAXV-t.
Figure 2. Using plasma mixtures of defined cFVII activity, rotational thromboelastometry was performed with human TF as the initiator. A graph showing velocity vs. time (A) and the bar chart of derived MAXV-t values (B) shows that MAXV-t decreases as cFVII activity increases. In a graph of CT vs. FVII activity (C), CT decreases as cFVII activity increases.
Figure 3. Determination of efficacy in the FVII deficient dog treated with AAV serotype 8 expressing cFVII. AAV-cFVII was administered at day 0 (vertical dotted line) and samples were collected at various timepoints. Normal canine plasma was given prophylactically in the first few days after AAV administration (red arrows). A prothrombin time assay (A) and a ROTEM assay (B) were used to monitor cFVII expression. Two ROTEM parameters are shown in (B), clot time (CT) and MAXV-t. A green line signifies the value of a hemostatically normal dog for each assay.