Functional Characterization of Rainbow Trout (*Oncorhynchus mykiss*) Chemokine 2 (CK-2)

by

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Abstract

Chemokines are cytokines with chemoattractant ability, and comprise one of the major groups of molecules in immune system. These are small, secreted proteins cause the migration of leukocytes to the sites of injury. Over 40 mammalian chemokines have been identified to date, and they have been implicated in a number of immune mediated processes, including regulation of inflammation, antigen presentation, blood cell development, metastasis, viral infection and wound healing. In rainbow trout, there have been fewer chemokines reported and only one functional study has been published. Rainbow trout chemokine 2 (CK-2) is the only known CC chemokine with a mucin stalk, which has the potential for extensive *O*-glycosylation. However, no functional characterization has been performed on this molecule yet. CK-2 shares the presence of a mucin stalk with the mammalian chemokines, fractalkine (CX_3CL1), lymphotactin (XCL1), and CXCL16. Another related trout CC chemokine sequence, CK-2.1, has been discovered recently, which has 98% nucleotide sequence identity with CK-2. CK-2.1 was believed to be a separate gene due to its apparent differential regulation in challenged rainbow trout. The question remained, however, whether or not CK-2.1 was a separate gene or an allele of CK-2. The goal of this project was to further characterize both CK-2 and CK-2.1.

Through genomic PCR on several outbred individuals it was shown that CK-2.1 is an allele of CK-2 but not a separate gene. Reverse transcriptase (RT) PCR analysis revealed an increased level of transcript both CK-2 and CK-2.1 in response to phytohaemagglutinin (PHA) stimulation of head kidney leukocytes (HKL) and peripheral blood leukocytes (PBL) collected from fish with different allelic distributions. Similar results were also observed in the rainbow trout macrophage/monocyte cell line, RTS11. Moreover, an anti-CK-2 antiserum was developed in rabbits, which cross-reacted with CK-2.1. This newly produced antibody was used to determine the protein expression levels in PHA stimulated rainbow trout tissues. RT-PCR was also performed on the same tissues in order to examine the transcript expression. Rainbow trout with both CK-2 and CK-2.1 were used for this experiment. An overall decreasing pattern of transcript (both CK-2 and CK-2.1) was observed in brain and HK over 24 hours, while protein was still detected at 24 hours post stimulation. However, in spleen the CK-2 transcript showed a slight upregulation at 4 hours post stimulation along with a very little or no CK-2.1 expression, although no protein was detected in spleen. Liver showed a very low level of CK-2 and CK-2.1 transcript at 8 hours post stimulation; while protein was again detected at 24 hours post stimulation. In addition, the sizes of the proteins found in different tissues were larger

than expected (≤30 kDa for CK-2 or ≤35 for CK-2.1), perhaps due to the presence of extensive *O*-glycosylation at the mucin stalk of the protein.

A chemotaxis assay was carried out, which is the definitive assay for chemokine activity. This assay showed migration of peripheral blood leukocytes across a membrane with 5μm pores toward CK-2 at an optimal concentration of 500ng/ml (17nm). Moreover, by pre-treating the recombinant chemokine with the polyclonal antisera, it was shown that the chemokine was actually causing the chemotactic activity. Pre-treatment of the cells with pertussis toxin, an inhibitor of G-protein signalling inhibited the migration of PBLs, established the fact that CK-2 caused chemotaxis by binding to a 7 transmembrane, G-coupled receptor just like all other known chemokines. Interestingly, CK-2 was also shown to attract RTS-11 cells.

Overall, the above findings indicate that CK-2 is functionally a chemokine with two very different alleles in rainbow trout. It is probably heavily *O*-glycosylated and different tissues express different sizes of the protein. This is only the second functional study of a fish chemokine.

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Chapter 1
Introduction

1.1 An Overview of the Immune System

The immune system provides a complete defense mechanism for an individual by protecting it from any potentially pathogenic microorganisms. The innate and adaptive responses are the two major components of the immune system. The innate immune system is considered to be the first line of defense against any kind of infection (Secombes *et. al*. 2001). This is a non-specific system that an individual is born with. It comprises many different immune cells and mediators; and takes care of many invading pathogens in a healthy individual. On the other hand, the adaptive immune system is highly specific and diverse, and generates specific antibodies and T-Cell receptors against a particular pathogen. Moreover, the adaptive system can give rise to memory and has the ability to distinguish self from non-self. The innate response precedes the adaptive response. It activates and determines the nature of the adaptive response and co-operates in the maintenance of homeostasis; therefore, innate immunity is an essential requirement for the adaptive immune response to work efficiently (Fearon and Locksley 1996; Fearon 1997). The antigen-specific lymphocytes of the adaptive immune response are activated by co-stimulatory molecules that are induced on cells that belong to the innate immune system during their interaction with micoorganisms. Furthermore, the cytokines produced during these early phases also play an important role in stimulating the subsequent adaptive immune response. In short, both innate and adaptive responses work together to successfully handle almost every possible infection in order to provide a complete and effective immune response.

1.2 Chemokines

Cytokines are the group of immune molecules responsible for regulating and initiating the inflammatory processes (Secombes *et. al*. 2001); and these molecules usually are released in response to an activating stimulus. Chemokines are a class of cytokines possessing the ability to attract leukocytes to the site of injury or infection (Lally *et. al*. 2003; Laing and Secombes 2004). They are an essential part of both the innate and adaptive immune system. These are small, secreted proteins and have been implicated in several immune mediated responses, such as, inflammation, intercellular communication, antigen presentation, blood cell development, metastatis, viral infection, and wound healing (Adler and Rogers 2005; Rossi and Zlotnik 2000; Baggiolini 1998; and Cyster 1999).

Diseases like inflammation and autoimmunity occur due to the improper functioning of the chemokine-mediated signalling system (Fernandez and Lolis 2002). Moreover, recently it has been postulated that chemokines play important roles in the development of nervous system. They have roles in neural progenitor cell migration (Knaut *et. al.* 2005) and axon guidance (Liebram *et. al.* 2005). Also, Alder and Rogers have proposed chemokines as a third major system in brain (Adler and Rogers 2005).

The protein product of chemokines usually ranges from 60-80 amino acids with basic properties (the isoelectric point is usually higher than 7; for example the pI of CK-1 is 10.7; Cyster 1999; Baggiolini 1998 and Vaddi 1997). Chemokines signal through specific receptors at leukocyte surfaces (Dixon *et. al*. 1998). These receptors belong to Rhodopsin family as they have seven transmembrane domains and are coupled to heterotrimeric GTP binding protein (G-proteins; Cyster 1999; Rossi and Zlotnik 2000). Induction of chemotaxis (migration of leukocytes toward the concentration gradient of chemokines) requires activation of pertussis toxin (PTX) sensitive G proteins (G_i); therefore, chemotaxis can be inhibited by PTX (Su *et. al.* 2001and Cyster 1999).

So far, about 50 different chemokines have been studied in mammals over the past few years (Laing and Secombes 2004). However, some members have been cloned in birds (Kaiser *et. al.* 1999, Hughes *et. al.* 2001), in amphibians (Braun *et. al.* 2002), and in fish (Dixon *et. al.* 1998; Fujiki 2002; Laing 2002). A few viral (Zou *et. al.*1999, Penfold *et. al.* 1999, MacDonald *et. al.* 1997) and bacterial (Rao *et. al.* 2002) chemokines have also been reported.

1.3 Structure of Chemokines

Typically, chemokine genes share a similar structure consisting of three exons and two introns (Graves 1995). Chemokines usually contain four conserved cysteines, which form two essential disulphide bonds. Each of these bonds involves a cysteine residue located near the amino-terminus; therefore, one amino terminal cysteine forms a disulphide bond with a cysteine placed centrally within the sequence (cys-1 to cys-3), while the second cysteine close to the amino-terminus forms a disulphide bond with a cysteine placed closer to the carboxyl terminus (cys-2 to cys-4) (Baggiolini 1998; Lewis *et. al.* 1995) (Figure 1.1). Nonetheless, exceptions to this rule are also possible because chemokines exist with only two cysteines or with six cysteines.

Figure 1.1: Structures of Chemokines. A schematic diagram showing the structure of the different classes of chemokines found in mammals. (Picture was not drawn in scale). TM, Transmembrane domain; Cyt, Cytoplasmic tail. C, Cysteines; X, any amino acid other than cysteine.

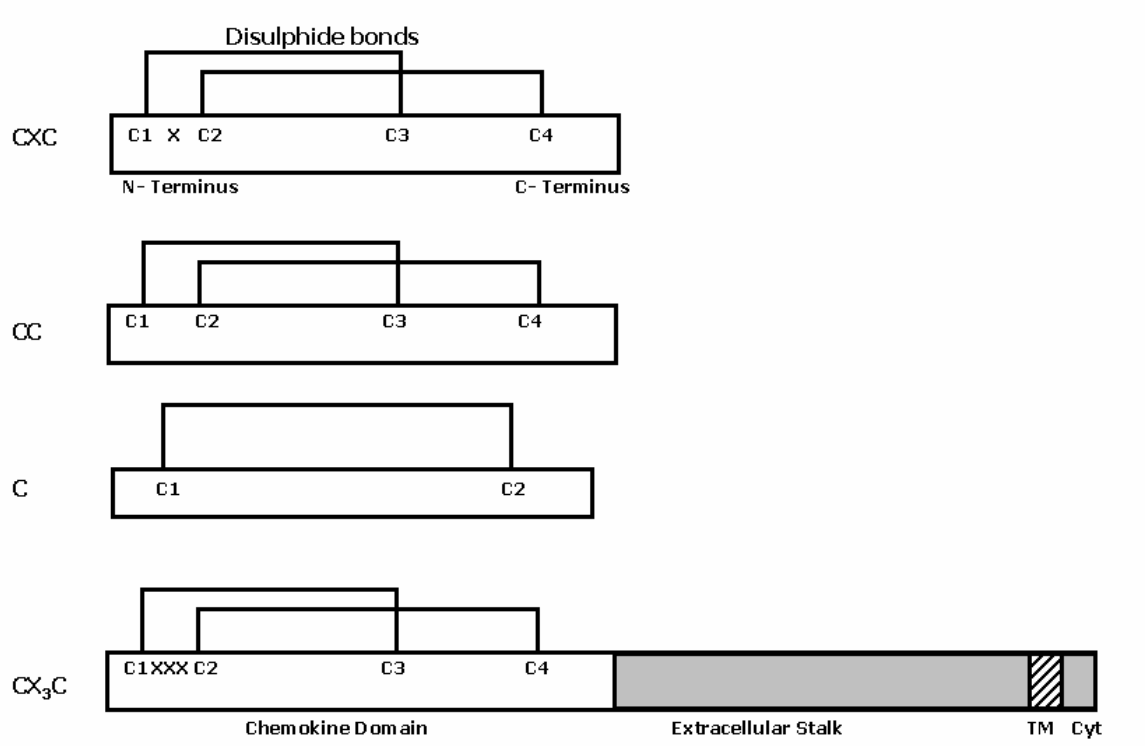

Disulphide bonds
CXC
C1 X C2 C3 C4
N- Terminus
C- Terminus
CC
C1 C2 C3 C4
C
C1 C2
CX3C
C1XXX C2 C3 C4
Chemokine Domain
Extracellular Stalk
TM
Cyt

Figure 1.2: Families of chemokines. All chemokines are grouped into four different families depending on the number of amino acids present between the *N*-terminal cysteines. The CXC or α family contains only one amino acid between the two cysteines on the amino terminus. The CC chemokine family has two adjacent *N*-terminal cysteines. The third family is the, C or γ chemokine family contains a single amino terminal cysteine, which forms one disulfide bond with the *C*-terminal cysteine. Finally there are the CXXXC or δ chemokines, where the first two cysteines are separated by three amino acids. These are tethered directly to the cell membrane via a long mucin stalk and induce both adhesion and migration of leukocytes. Structurally, the CXC, CC, C and CXXXC families are quite similar. They all have short amino-terminal domain preceding the first cysteine, followed by a backbone of three anti-parallel beta sheets. An alpha helix is found near the *C*-terminus. The picture was adopted from http://www-ermm.cbcu.cam.ac.uk (the structure of C chemokine was drawn using the description).

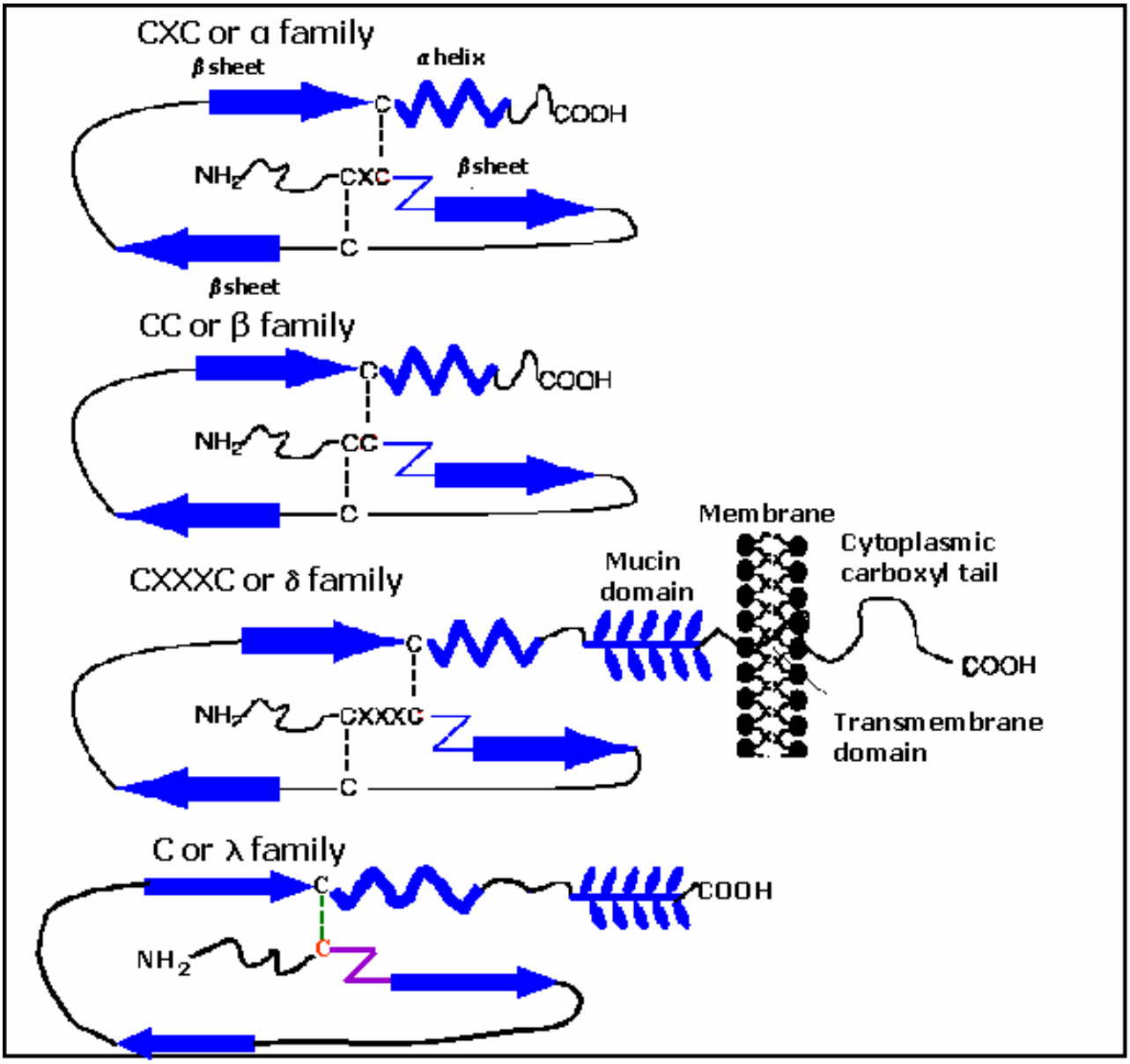
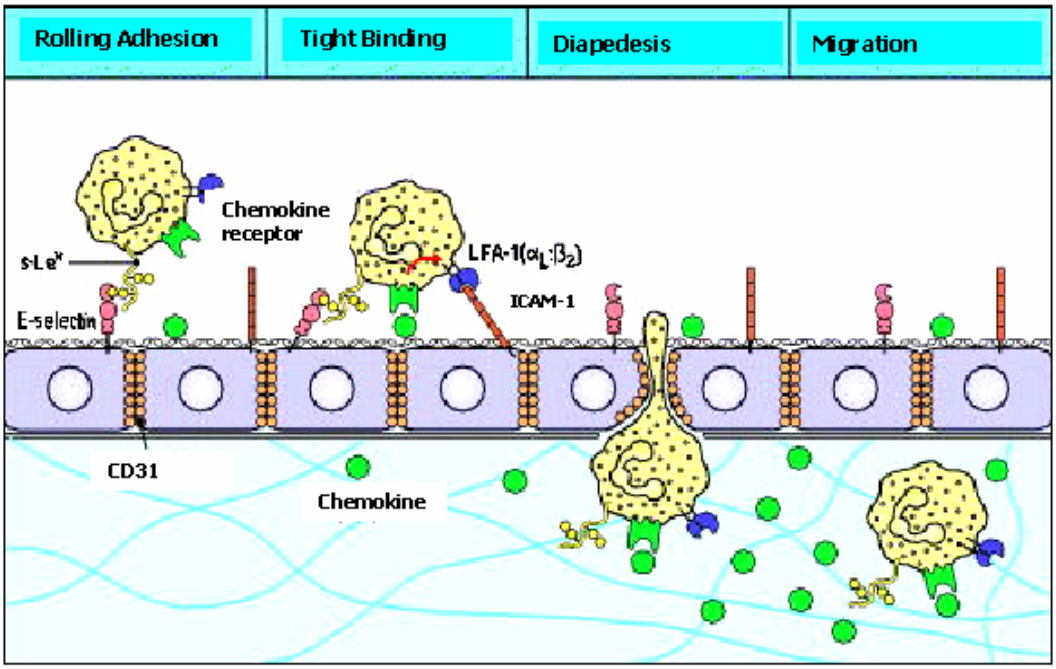

CXC or α family
β sheet
α helix
COOH
NH2
CXC
β sheet
β sheet
CC or β family
COOH
NH2
CC
CXXXC or δ family
Membrane
Mucin domain
Cytoplasmic carboxyl tail
COOH
NH2
CXXXC
Transmembrane domain
C or λ family
COOH
NH2

Figure 1.3: Leukocyte trafficking. A major function of chemokines is to promote leukocyte trafficking. One of the earliest cells to be involved in repairing injury are neutrophils. During injury, chemokines get secreted from the injury site. The trafficking occurs as a step-wise manner. First of all, the leukocytes reversibly bind to the lumenal surface of endothelium through selectins and their carbohydrate ligands on the leukocytes (E-selectin and s-Le^x). Further binding occurs between endothelium ICAM and leukocyte integrins to provide stronger interaction. Also, chemokines bind to the specific receptors on the leukocytes and remodel cytoskeleton. These cause changes in cell shape and then leukocytes squeeze though the endothelium, a process known as diapedesis. Once the leukocytes are out of the circulation chemokines further guide them to reach to the site of infection or injury (Janeway 2005).


Rolling Adhesion
Tight Binding
Diapedesis
Migration
Chemokine receptor
s-Le^x
E-selectin
LFA-1(α_L:β_2)
ICAM-1
CD31
Chemokine

Structurally, chemokines are composed of a short amino-terminal domain preceding the first cysteine, followed by a backbone of three anti-parallel beta sheets; connecting loops are found between second and fourth cysteine. Moreover, an alpha helix is found near the carboxyl terminus (Dixon *et. al*. 1998; Baggiolini 1998) (Figure 1.2). Although chemokines tend to form dimers and trimers they function as a monomer. There are two sites that are responsible for binding to the receptor. The primary site is located at the NH_2 –terminal, and a secondary site is located in the flexible loop region that follows the second cysteine (Lewis *et. al.* 1995).

1.4 Families of Chemokines

Chemokines are classified into four families depending on the motif displayed by the first two *N*-terminal cysteines, since most of them have four characteristic cysteines (Rossi and Zlotnik 2000). The CXC or α family has a single amino acid between the two cysteines on the amino terminus. Most CXC chemokines are chemoattractants for neutrophils. When the first two *N*-terminal cysteines are adjacent it is called a CC or β family chemokine. CC chemokines generally attract monocytes, lymphocytes, basophils, eosinophils, and natural killer (NK) cells (Dixon *et. al*. 1998; Vaddi 1997, Loetscher *et. al.* 1996). CC chemokines can be further divided into a group containing six cysteines; thereby, forming three disulphide bonds. Depending on the positioning of the extra cysteines they can be called C6-β or 6-C-kines. 6-C-kines have two additional cysteine residues after the 4 consensus chemokine cysteines at the C-terminus; therefore, an additional disulphide bond can be found in between the 5^{th} and 6^{th} cysteine residues (Hedrick and Zlotnik 1997). On the other hand, C6-β has one extra cysteine between the second and the third cysteines, and another after the fourth cysteine; hence, there is an additional bond between 3^{rd} and 6^{th} cysteine (Wang *et. al.* 2000*)*. The third family is the CXXXC or δ chemokines, where the first two cysteines are separated by three amino acids. The CX_3C family has only one member named CX_3CL1 or fractalkine (Dixon *et. al*. 1998; Vaddi 1997). This family is quite interesting since it has a mucin stalk and a transmembrane region at the carboxyl terminal. Therefore, this is a chemokine that is membrane bound, but it can also function as a secreted form when transmembrane domain is cleaved (Bazan *et. al.* 1997). These are tethered directly to the cell membrane via the long mucin stalk and induce both adhesion and migration of leukocytes. Nevertheless, Pan *et. al.* found the similar molecule in murin brain and named it as neurotactin (Pan *et. al.* 1997). Fractalkine is a known chemotactic factor for monocytes/macrophages as well as NK cells and T lymphocytes (Gevrey *et. al.* 2005). Finally, the C or λ chemokine family seems to be lymphocyte specific. This specific class contains only one *N*-terminal cysteine, which forms one

disulphide bond with the carboxyl terminal cysteine. Although this C chemokine is a little different it still maintains structural similarity with CC, CXC, and CX3C chemokines, but it has a mucin stalk at the carboxyl terminus (Kelner *et. al.* 1994; Kuloglu *et. al* 2001) (Figure 1.2). Lymphotactin is the sole member of the C subgroup.

In addition, the CXC family is subdivided into two groups based on the presence of an ELR amino acid motif preceding the first cysteine: the ELR-CXC chemokines and the non-ELR-CXC chemokines. Furthermore, functionally chemokines fall into two major categories, one being homeostatic that are produced and secreted constitutively; and another one is inducible that are produced by cells during infection or stimulus (Laing and Secombes 2004). Homeostatic chemokines are generally involved in lymphocyte trafficking, immune surveillance and localization of lymphocytes with antigen in the lymphatic system. On the other hand, inducible ones prompt migration of leucocytes to an injured site during infection. They also activate cells to raise an immune response and initiate wound healing process (Laing and Secombes 2004).

1.5 Leukocyte Trafficking

One of the major functions of chemokines is to help in leukocyte trafficking. Both host defence and immune surveillance are extremely dependent on mobile leukocytes, which form continuously improved microenvironments and can be rapidly recruited to the site of injury or inflammation (Mitra *et. al.* 2004). This event is known as chemotaxis, migration of leukocytes toward a chemical gradient (Downey, 1994). Chemotaxis is vital to many biological processes of eukaryotic cells, including migration of macrophage and neutrophils during wound healing, homing of thymocytes, migration of neural crest cells, and the aggregation of *Dictyostelium* cells to form a multicellular organism (Devreotes and Zigmond 1988). Usually, the molecular control of this trafficking requires two major classes of molecules: various cell adhesion molecules, especially integrins and selectins, and leukocytes chemotactic factors such as, chemokines (Springer 1994). The heparin binding properties of chemokines enable them to be retained in the extracellular matrix or by cell-bound proteoglycans so that they can interact with the rolling leukocytes. Actually, these chemokines bind to glycosaminoglycans (GAGs) of the heparin and heparan sulfate (HS) family. GAGs are highly acidic and linear polysaccharides widely distributed in the extracellular matrix and on cell surfaces. This binding is favourable for chemokines for being closer to the sites of release since they are small and readily diffusible proteins (Imai *et. al.* 1997; Mummery and Rider 2000). Chemokines interact with the specific receptors on the leukocyte surface and initiate the process. The most impressive effect

during this trafficking is the shape change of the leukocytes. This occurs due to the interaction with chemokines, which induces remodelling of the cytoskeleton (Vaddi 1997) and helps leukocytes move though the endothelium. Also, stimulation induces the upregulation and activation of integrins causing the leukocytes to adhere to the endothelial cells of the vessel wall before migration (Springer 1994). Moreover, several rapid and transient events occur during the leukocyte trafficking, including, the increased intracellular free calcium concentration, the production of microbial oxygen radicals and bioactive lipids, and the release of the contents of the cytoplasmic storage granules (Baggiolini *et. al.*1998 and Baggiolini *et. al.*1997). As soon as the leukocytes are outside of the circulation, they again utilize chemokines as signals that guide them to the tissue they need to reach, such as inflammatory lesions (Vaddi 1997).

1.6 Teleost Immune System

The organization of the teleost fish immune system is quite different from mammals. The major difference is that fish lack bone marrow and lymph nodes, instead they use kidney as a major lymphoid organ. Besides, they have thymus, spleen, and mucosa-associated lymphoid tissues (Press and Evensen 1999). The cell population that is responsible for both innate and adaptive immune responses includes lymphocytes, monocytes, macrophages, granulocytes, and thrombocytes. Also, teleosts have mast cells, non-specific cytotoxic cells, and dendritic cells (Press and Evensen 1999). Apparently, the innate immune system is of primary importance in combating infections in fish since the adaptive immune system is not that efficient due to its evolutionary status and poikilothermic nature. Therefore, the adaptive system has a limited antibody repertoire, affinity maturation and memory, and slow lymphocyte proliferation. Thus, the adaptive immune system of fish is sluggish compared to the instant and relatively temperature independent innate immune response (Alexander and Ingram 1992; Ellis 2001; and Du Pasquier 1982).

Over the past few years, many fish immune genes have been isolated and have shown compelling evidence indicating the homology between teleost and mammalian immune systems. The most important ones are the cloning of teleostean major histocompatibility (MH) genes along with immunoglobulin genes and T-cell receptors (TCR). Both MH I and MH II genes have been cloned in teleosts and they exhibit typical levels of polymorphism seen in mammals (Dixon *et. al.* 1996). Moreover, some additional molecules involved in antigen presentation (invariant chain and calreticulin) have been cloned (Fujiki *et. al.* 2003, Kales *et. al.* 2004) in rainbow trout. All four TCR genes have been isolated recently (Nam *et. al*. 2003) along with accessory molecules including CD4

(Hansen 2000) and CD8 (Dijkstra *et. al.* 2006). Again, the complete sequence of the immunoglobulin heavy-chain gene has been cloned in teleost (Amemiya and Litman 1990).

Interestingly, fish possess a network of signalling molecules, such as, cytokines and chemokines, which control and coordinate the innate and adaptive immune systems (Secombes *et. al.* 2001). Progress in the discovery of fish chemokines has accelerated in recent years, but still not too many fish chemokines have been discovered (Liu *et. al.* 2002). All of the fish chemokines that have been reported to date belong to CXC and CC families, and equivalents of the C and CXXXC have not been discovered yet (Dixon *et. al.*1998; Long 2000; Laing 2002; and Liu *et. al.* 2002). The first reported chemokine belonged to CC family, and it was named CK-1 (Dixon *et. al.*1998). Recently, more CC chemokine genes have been discovered making the total number of CC chemokine in trout 18. Some of these recent chemokines (CK4, CK5. CK7, CK8, CK12) have been found as pairs (a and b); therefore, still it is not certain if they are alleles or separate genes (Laing and Secombes 2004). In addition, 26 CC chemokines have been identified in catfish (Peatman *et. al.* 2005). Also, a highly inducible CC chemokine has been isolated in lipopolysaccharide stimulated rainbow trout macrophages, which is similar to human CCL4 (MacKenzie *et. al.*2004).

Several CXC chemokines have been identified in fish, including a CXCL8 (IL-8) like molecule in lamprey (a jawless fish or aganatha) (Najakshin *et al.* 1999), which is one of the earliest chordates. CXC chemokines, similar to the IL-8 gene, have also been found in flounder (Lee *et al.* 2001), in rainbow trout (Laing *et al*. 2002; Sangrador-Vegas *et al*. 2002), in carp (Savan *et al.* 2003), zebrafish (Long *et al.* 2000) as well as, three catfish CXC chemokines (Baoprasertkul *et. al* 2005). To date only one functional study has been published, however (Lally et al., 2003).

There are many chemokines identified in mammals; however, the discovery of fish chemokines is promising and the presence of fish chemokines representing several structural groups suggests that the chemokine network in fish may be as extensive as the one found in mammals.

1.7 The Study of Rainbow Trout CK-2

Rainbow trout CK-2 is the second CC chemokine that has been reported in fish. CK-2 is a member of the CC chemokine family and possesses all the required characteristic features. It has two adjacent cysteines at positions similar to the known CC chemokines with two more cysteines at positions 59 (central) and 73 (*C*-terminal). Also, the cDNA has six AUUUA repeats within the 3' untranslated region (Liu *et. al.* 2002). These repeats are usually found in 3'-untranslated region of genes encoding

chemokines, cytokines, and oncogenes (Bakheet *et. al.* 2001). The function of this AUUUA repeat is to facilitate the degradation of mRNA by RNase E (Wennborg *et. al.* 1995) and that allows the production of cytokines to be strictly controlled by particular induction signals (Dixon *et. al.* 1998). The carboxyl end of CK-2 consists of an extra long section rich in serines, prolines, and leucine suggesting that fact that CK-2 has a mucin stalk at the end of carboxyl domain along with an amino terminal chemokine domain (Liu *et. al.* 2002). As discussed earlier, a mucin stalk is usually a characteristic of CX_3C (fractalkine) subclass. Usually, a mucin stalk region is identified by extensive *O*-glycosylation of serine and threonine residues located in short tandem repeats of varying length and by their extended backbone structure (Bazan *et. al*. 1997). However, a member of the mammalian CXC chemokine family, CXCL16, has been also shown to posses a mucin stalk (Wilbanks *et. al.* 2001). The difference between the trout and human stalks is that the trout stalk does not contain a transmembrane domain at the carboxyl end of the molecule. Also, the trout mucin stalk is only half the length of the known mammalian stalks (Liu *et. al.* 2002). Having a transmembrane domain suggests the fact that the mammalian molecules can be membrane bound and suspended by the stalk (Wilbanks *et. al.* 2001). Therefore, assumption is that CK-2 might function similarly to the cleaved or truncated mammalian fractalkine (Liu *et. al.* 2002). However, a recent study has shown that lymphotactin (C chemokine) also has a glycosylated mucin stalk, and this chemokine does not have a transmembrane domain (Dong *et. al.* 2005). Lymphotactin has been shown to be produced by NK cells, and they can attract both NK cells and T lymphocytes (Hedrick *et. al.* 1997). Thus, it could be predicted that CK-2 might show some similar function like mammalian lymphotactin.

The structure of fractalkine suggests that it can function on endothelium while it is being presented on its own stalk, which can promote the transient activation and adhesion of monocytes and T-cells to the endothelium. Furthermore, cleavage of fractalkine might function as regulatory mechanism to modulate trafficking at local sites (Bazan *et. al*. 1997). It has been proposed that only a few chemokines are expressed constitutively in the brain at detectable levels; and that fractalkine is one of them. Fractalkine has been shown to be expressed constitutively in human, rat, and mouse brain; also, it can be present in two distinct forms such as, as a membrane-bound protein with properties of cell adhesion or as a soluble molecule, which is obtained through proteolytic cleavage, that exhibits chemotactic features (Bazan *et. al.*, 1997; Chapman *et. al.*, 2000). Moreover, Chapman *et. al.* (2000) demonstrated that proteolytic cleavage of fractalkine from neuronal membranes occurs in response to neurotoxic insult; which leads to chemotaxis of primary microglial and monocytic cells and represents an early event in the inflammatory response to neuronal injury.

Until now, rainbow trout CK-2 is the only CC chemokine containing a mucin stalk in any organism. The full-length trout CK-2 is 25% identical to human fractalkine and has a very minimal similarity to CXCL16. The chemokine domain of CK-2 has 20% identity with another rainbow trout CC chemokine, CK-1 and 44% identity to the known carp CC chemokine (Liu *et. al.* 2002). Since CXCL16 is a member of the CXC chemokine family, it has little similarity with both the CC and CXXXC families. This chemokine is expressed on the surface of antigen presenting cells and is a second example of a chemokine with a transmembrane domain. Also, this chemokine has been shown to play dual role in attracting activated lymphocytes during inflammation and facilitating immune responses via cell-cell contact (Wilbanks *et. al.* 2001). On the other hand, CK-1 is a member of the CC-chemokine family and has been shown to attract rainbow trout peripheral blood leukocytes (Lally *et. al.* 2003).

Moreover, Southern blot analysis revealed that there might be two copies of CK-2 gene in trout. Also, northern blot analysis revealed strong expression of CK-2 in phytohaemagglutinin (PHA) stimulated head kidney leukocytes and peripheral blood leukocytes, but not in unstimulated cells. The protein product is about 20.8 kDa with a 191aa open reading frame (Liu *et. al.* 2002).

In addition to CK-2, another chemokine sequence from trout has been identified in our lab, which has 98% nucleotide sequence similarity with CK-2 and was named CK-2.1. Structurally CK-2.1 is almost identical to CK-2 with an exception of a single amino acid difference in the mucin stalk, a tryptophan to arginine substitution at position 218 of the CK-2.1 protein, and an extra segment of mucin stalk containing 42 additional amino acids (Appendix C, Figure C1). The protein product of CK2.1 is around 25kDa. Despite having almost identical structure to CK2, CK-2.1 showed differential regulation in response to stimuli suggesting a different function; therefore, it was believed that CK-2.1 was a separate gene. However, the question of whether or not CK2.1 is a different gene or an allele of CK-2 has not been adequately addressed to date.

1.8 Objective of the Research

The objective of this research project is to explore the function of CK-2 and CK-2.1 in rainbow trout. CK-2 is the only CC chemokine with a mucin stalk, and CK-2.1 is 98% similar to CK-2. Therefore, it is worth studying these two related molecules in order to figure out if they are separate genes or alleles. Genomic PCR and Southern blot analysis will be performed to answer this question. Moreover, the transcript and protein expression will also be examined in both *in vivo* using

challenged rainbow trout and *in vitro* using stimulated rainbow trout cell lines. In order to detect the protein level, polyclonal antibodies will be developed since no commercial antibody is available. Lastly, the functional assays will be carried out to study the chemotactic ability of CK-2 using PBLs and RTS-11.

Over the past few years, tremendous efforts have been made to identify fish chemokines. The chemokines are one of the most important immune molecules and they have various important functions as found in many mammalian studies. Therefore, it is worth studying these molecules in fish, which would provide a great deal of information in aquaculture. In addition, the chemokine in this study has some similarities with mammalian chemokines, which makes it quite interesting to study. More detailed knowledge from studying this molecule might help designing a vaccine for the aquaculture using chemokines as immunoadjuvants.

Chapter 2
Materials and Methods

2.1 Genotyping fish

2.1.1 Collection of Blood Samples

Rainbow trout were anaesthetized with 0.01% ethyl 3-aminobenzoate methanosulfonate salt (MS 222; Sigma-Aldrich, ON, Canada) and 0.1ml blood was drawn from the caudal blood sinus with 23G needle. The blood sample was mixed with 10ml 1X phosphate buffered saline (PBS) (137mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4. $7H_2O$, and 1.4 mM KH_2PO_4; pH 7.4) and centrifuged at 1600rpm for 5 minute (min) at room temperature (RT). After centrifugation, the supernatant was discarded and the blood cells were washed 2 times with PBS. At the end, the blood cells were re-suspended in 1ml PBS. The concentration of the blood sample was measure by diluting 10µl blood sample in 1.49 ml of dH_2O and absorbance was measured at 541nm using UV-160 Spectrophotometer (Mandel Scientific Co., ON, Canada). The concentration was calculated by multiplying the absorbance by 10^{10} /4.4, which gave cells/ml.

2.1.2 Extraction of Genomic DNA

A cell suspension containing $5x10^7$ cells was placed into a microcentrifuge tube. The cells were mixed with 500µl denaturing solution (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 20 mM EDTA) containing 25µl 10% SDS, and 2.5µl proteinase K (0.1 mg/ml). The mixture was incubated at 55^0C overnight. After the incubation, 500µl saturated neutral phenol was added and mixed vigorously followed by centrifugation at 13,000rpm for 3 min. The top layer was moved to another microcentrifuge tube and to that 500µl PCI (Phenol, chloroform, isoamayl alcohol) was added, followed by vigorous vortexing and centrifuging at 13,000rpm for 3 min. The top layer was transferred to a new tube, 500µl of CIAA (Chloroform : iso-amylalcohol = 24:1) was added and again centrifuged at 13,000rpm for 1 min. Again, the top layer was taken out and added to a separate tube to which 1/10 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol were added. The tube was inverted few times and stored at -80^0C for 30 min. After 30 min, the tube was centrifuged at 15,000rpm for 15 min. The white DNA pellet was kept while all the liquid was discarded, and the DNA was washed twice by 70% ethanol. After air-drying, the pellet was re-suspended in TE buffer

(10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.8). Again the concentration was determined by measuring the absorbance at 260nm.

2.1.3 Polymerase Chain Reaction (PCR)

Several different primer sets were used to amplify the region representing the only difference between CK-2 and CK-2.1. Therefore, the primers were designed so that they could span the mucin region. A 20µl PCR reaction was set up as follows, 2µl of 10x LA PCR buffer II, 1.6µl of 2.5mM dNTP mixture, 1µl of each primer, 1µl of 50ng/µl template, and 0.1µl of LA Taq (Takara, Japan). Forward CK-2 gDIG sense: 5' GCAGAAAAGCTGGTGTCGTG (Tm 57°C) and reverse CK-2 antisense (Tm 57°C) were used as one pair. Forward CK-2 stalk sense: 5' GTCTGAGCTCAACATTTCTC (Tm 53°C) was used with several reverse primers such as, CK-2 stalk antisense: 5' AGGAGCTTCAGCCATTAGCA (Tm 53°C); CK-2 SP2: 5' GTTCTACAAGCCCCCATAAG (Tm 55°C); and CK-2 SP4: 5' ATGGGCACATACAATACTGG (Tm 54). The thermal cycler conditions were as follows: 95°C for 5 min, 30 cycles of 95°C for 30 seconds (sec), Tm for 30 sec and 72°C for 1 min, then 72°C for 5 min. The PCR product was run on 1.5% agarose gel containing 0.1% ethidium bromide (EtBr) and the DNA bands were visualized with a UV transilluminator containing an EtBr filter. The image system was a Fluorochem 8000 imager, which used Alpha Innotech (San Leandro, CA, USA) visible imaging software.

2.2 Southern Blotting

2.2.1 Collection of Blood Samples

Blood samples from three different rainbow trout with three different allelic distributions were collected as described in section 2.1.1.

2.2.2 Extraction of Genomic DNA RE Digest

For Southern blotting, DNA was extracted from $3.5x10^7$ red blood cells using Qiagen's Dneasy Tissue Kit (Qiagen, ON, Canada). The concentration was determined by measuring the absorbance at 260 nm using UV-160 Spectrophotometer. Three aliquots of 10µg DNA were digested to completion at 37^0C for 12 hr with 20U of either *Pst*I, *Eco*RI, or *Hind*III. The digests were purified by PCI and CIAA extraction; followed by precipitation with 3 M sodium acetate and 100% ethanol at -80^0C for

30 min as described above. Afterwards, the samples were washed by 70% ethanol and resuspended in TE.

2.2.3 Southern Blotting

The purified digested DNA was electrophoresed on 1% agarose gel at 20V for 8.5 hr. The gel was stained in 1ug/ml EtBr for 30 min and visualized on a Fluorchem 8000 imager (Alpha Innotech) to check the digestion. Then the gel was denatured for 15min in 0.25N HCl. After rinsing with deionized water, the gel was incubated twice in 0.2 N NaOH and 1.5 M NaCl buffer for 15 min. Again, the gel was washed with deionized water and incubated in 0.25 M Tris-HCl and 1.5 M NaCl buffer for 15min twice.

The transfer of the DNA to a membrane was set up by filling the Mupid 21 with 10x SSC, placing a plastic plate over the top, followed by a piece of filter paper soaked with 10x SSC and cut to fit over the plate and into the buffer. A piece of filter paper was slid under the gel in the 0.25 M Tris-HCl and 1.5 M NaCl buffer so that it can be transferred to the plastic plate. A previously cut nylon membrane, the same size as the gel, was rinsed in MilliQ water, equilibrated in 10x SSC, and then placed on top of the gel, followed by 3 gel size filter papers and plastic wrap to prevent buffer bypass. A glass rod was used to roll over the top of the membrane to remove the air bubbles between the gel, the plate and membrane. Several inches (~5 inches) of gel size paper towels were placed on top. A large plastic plate was secured over top of the transfer apparatus with a weight and the transfer of the DNA occurred by capillary action overnight.

After the overnight transfer, the lanes were marked on the nylon membrane with a pencil and then the DNA on the gel and membrane were visualized using the Fluorchem 8000 imager to ensure the complete transfer of DNA. The membrane was soaked in 2x SSC, air dried for 30 min, and DNA was cross-linked to the membrane under reflective UV light for 50 sec.

2.2.4 Preparation of Digoxigenin (DIG)-labeled Probe

The DNA probe was prepared by PCR-amplifying genomic DNA (50ng) using primers specific for chemokine domain and stalk region with some introns (forward 5'-GCAGAAAAGCTGGTGTCGTG-3'and reverse 5'-GCTCTCCATTCCAAACTCTC-3'). The PCR product was labeled using the DIG probe synthesis kit (Roche Diagnostics, Indianapolis, IN, USA). An unlabelled control was also included to ensure that labeling occurred. The PCR conditions were as follows: 95°C for 2 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, then 72°C

for 7 min. Both the control and labelled probes were run on a 1% agarose gel. The gel stained with EtBr and visualized under UV light.

2.2.5 Hybridization and Detection

Previously dried and cross-linked membrane was soaked in 2x SSC. The membrane was then pre-hybridized for 2 hr at 42^0C in a hybridization bottle (Fisher Scientific, ON, Canada) using hybridization buffer (5x SSC, 50 mM NaH_2PO_4 pH 7.0, 50% formamide, 2% blocking solution, 5.5% sarkosyl, 7% SDS) in a hybridization oven (Robbins Scientific). Following pre-hybridization, 12µl probe was added to 6ml of the hybridization buffer, boiled for 10 min, quenched on ice for 3 min, and then added to the membrane to hybridize overnight at 42^0C.

After hybridization, the membrane was subjected to following washes in order to remove any non-specific hybridization by the probe. The first wash step was with 2x wash solution (2x SSC, 0.1% SDS) at 37°C for 5 min followed by two washes with pre-heated 0.1x wash solution (0.1x SSC, 0.1% SDS) for 30 min at 65°C. The membrane was then soaked in DIG buffer 1 (10 mM maleic acid, 150 mM NaCl) and blocked in DIG buffer 2 (1% blocking reagent in DIG buffer 1) for 30 min. After blocking, the primary alkaline phosphate conjugated anti-DIG antibody was added at a dilution of 1:10000, the membrane was placed in a Kapak bag and incubated for 30 min. The membrane was then washed 7 times for 15 min with DIG buffer 1 containing 0.3% Tween 20. In order to detect the signal, the membrane was soaked in DIG buffer 3 (0.3 M Tris-HCl pH 9.5, 0.1 M NaCl, 55 mM $MgCl_2.H_2O$) for 5 min at RT. In the end, CDP Star reagent (Roche Diagnostics, Indianapolis, IN, USA) was added to the membrane and incubated at 37°C for 15 min. A Fluorchem 8000 (Alpha Innotech) was used to visualize the signals. The membrane was exposed for 20 min using chemiluminesence filter at medium sensitivity.

2.3 Construction of Expression Vector

In total, four recombinant proteins were expressed including; chemokine 2 (CK-2), chemokine 2.1 (CK-2.1), mucin stalk, and chemokine domain. The clones containing CK-2 and CK-2.1 were prepared previously; therefore, for these two proteins only expression was carried out. However, separate protein fragments of mucin stalk and chemokine domain were cloned into expression vector.

2.3.1 Primer Design and PCR

The full-length cDNA of CK-2 was already cloned in pRSET A by previous student; therefore, this clone was used to amplify the sequence specific to mucin stalk and chemokine domain. Primers for the mucin stalk protein included, 5' CGCCGGATCCATGGCCCGTCTGAGC (forward; Tm=47^{0}C) and 5' CGCCAAGCTTACTCTATTGGTTGGA (reverse; Tm=51^{0}C). Chemokine domain primers constructed were: 5' CGCCGGATCCGAAAAGCTGGTGTCGTG (forward; Tm=52^{0}C) and 5' CGCCAAGCTT TTCAAACTGATGAATCTTGC (reverse; Tm=52^{0}C) (Primers provided by Sigma-Genosys, ON, Canada). The thermal cycler conditions were as follows: 95°C for 5 min, 30 cycles of 95°C for 30 sec, 47°C (mucin domain) and 52°C (for chemokine domain) for 30 sec and 72°C for 1 min, then 72°C for 5 min. The PCR products were run on a 1.5% agarose gel containing 0.1% ethidium bromide (EtBr) and the DNA bands were visualized under UV light.

2.3.2 Preparation of Insert and Vector

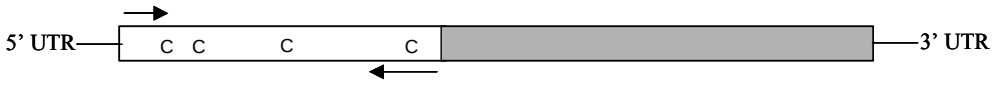
The amplified PCR products were purified by PCI and CIAA extraction method as described in section 2.1.2, and resuspended in H_20. This purified PCR products were digested with *Bam*H1 and *Hind*III for overnight at 37^{0}C. Again the digested fragments were purified by the above procedure (PCI and CIAA extraction). The digested and purified products were run on a 1.5% agarose gel. For the mucin stalk, a 311bp band was cut out of the gel and purified using an Agarose Spin Column (Sigma-Aldrich, ON, Canada). A similar procedure was followed for the chemokine domain, where a 183bp band was cut out of the gel and purified. Moreover, the vector (pRSET A) was also digested using the same restriction enzymes. About 10μg vector was digested with *Bam*H1 and *Hind*III for 2 hr at 37^{0}C. The digested vector was further purified by phenol chloroform extraction and run on the same gel with the insert. An approximately 3000bp band was cut out of the gel and purified using an Agarose Spin Column as described above.

2.3.3 Ligation and Transformation

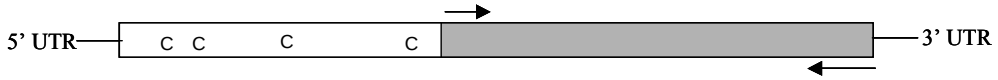
The ligation of the insert with pRSET A vector was carried out at 4°C overnight. Reagents from pGEM T-easy kit (Promega, MI, USA) were used for the ligation. In brief, 3.5μl of extracted insert, 50 ng vector, 4.5μl of 2x ligation buffer, and 3 units of T4 DNA ligase were added to the reaction.

Figure 2.1: Cloning of the chemokine domain and mucin stalk region. Schematic diagram showing the structure of the full-length CK-2 mRNA indicating the position of the primers for amplifying the chemokine domain and the mucin stalk regions. The arrows indicate the primer positions. A 311bp fragment was amplified for producing the mucin stalk. The sequence encoding just chemokine domain was also amplified by using the two primers flanking the domain. A 183bp fragment was amplified in this case. The 'C' denotes the positions of cysteines (not drawn in scale).

Chemokine domain

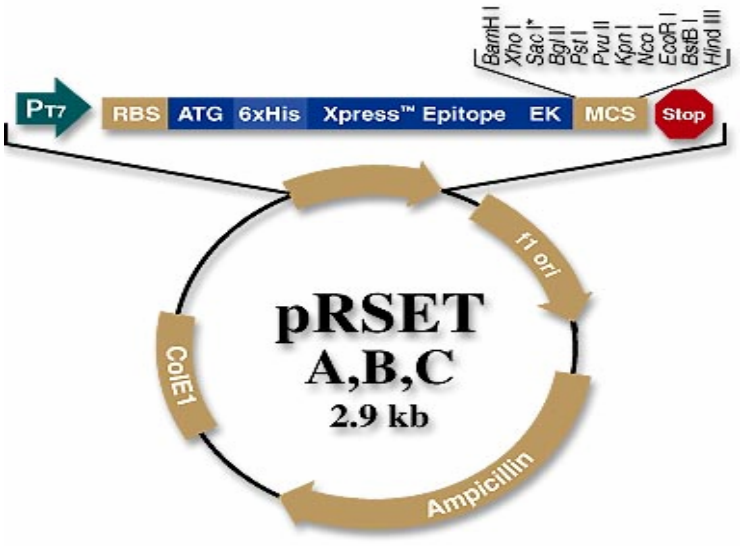


Mucin stalk



Chemokine domain Mucin stalk

Figure 2.2: Schematic diagram of the structure of the expression vector pRSET A. Several important features make this vector an ideal vector for protein expression. The vector contains T7 promoter, which gives a high level of protein expression. It has a sequence coding for a 6-histidine tag, which helps purifying protein through nickel-affinity chromatography. Moreover, the vector has an Xpress™ epitope for antibody (anti-express) recognition during Western blotting, and an enterokinase cleavage site to remove the 6-histidine tag using enzyme enterokinase. The multiple cloning site contains many different restriction enzyme cleavage sites which facilitates cloning of genes of interest. Finally, the vector carries gene for ampicillin resistance to help select *E. coli* cells possessing pRSET A plasmid (Figure adapted from www.invitrogen.com).


BamH I
Xho I
Sac I*
Bgl II
Pst I
Pvu II
Kpn I
Nco I
EcoR I
BstB I
Hind III
PT7
RBS
ATG
6xHis
Xpress™ Epitope
EK
MCS
Stop
f1 ori
pRSET
A,B,C
2.9 kb
ColE1
Ampicillin

After overnight incubation, the whole ligation mixture was used to transform into XL1 blue MRF' *E. coli* competent cells. The competent cells were mixed with the ligation mixture and left on ice for 30 min. The cells were then heat shocked for 45 sec at 42°C in an Isotemp 1006D (Fisher Scientific, Nepean, ON) water bath incubator, quenched on ice for 3min. and mixed with 250µl of SOC media (0.5% yeast extract, 2% tryptone, 10 mM sodium chloride, 10 mM magnesium chloride, 2.5 mM potassium chloride, 10 mM magnesium sulphate and 20 mM glucose). The cells were then incubated for 30 min. at 37^0C in a water bath with gentle shaking (200rpm). The transformed cells were then plated onto Luria Bertani (LB) plates containing Ampicillin (Amp) at a final concentration of 100ug/ml. Two plates were spread with 10% and 90% of the total volume of the transformation mixture. The plates were incubated at 37^0C for overnight.

2.3.4 Plasmid Purification and Sequencing

In order to isolate plasmids, Daniel's modified alkaline miniprep (Sambrook *et. al.* 1989) method was followed. Five colonies were chosen from the plates made after transformation. Five ml LB-Amp broth was inoculated with individual colonies and incubated overnight at 37^0C. After incubation, 1.5 ml of the bacterial culture was centrifuged and resuspended in 100µl of Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). After 5 min, the cells were lysed with 200µl of Solution II (1% SDS and 0.2N NaOH) for 5 min on ice. The cells were then neutralized by adding 150µl of Solution III (3 M potassium acetate and 11.5% (v/v) acetic acid). The reaction mixture was centrifuged after 5 min and supernatant was collected. To the supernatant, about 20 µg RNAse A was added to remove any contaminating RNA. The next step was to remove contaminating proteins and genomic DNA by PCI and CIAA extraction. The plasmid was precipitated with 1 volume of 2-propanol and centrifuged for 15 min. at 15,000rpm. Following centrifugation, the pellet was washed twice with 1ml of 70% ethanol and centrifuged at 15,000rpm for 2 min. Finally, the plasmid DNA was resuspended in de-ionized water. The concentration of the plasmid was determined using absorbance and 260 and 280 nm on a UV-160 Spectrophotometer.

For sequencing, the concentration of each extracted plasmid solution was adjusted to 0.1µg/µl in de-ionized water and submitted to the University of Waterloo molecular core facility. Derived sequences were compared with the published CK-2 sequence (Fujiki *et al.* 2002). Also, the sequences were checked for any error and aligned with the full CK-2 using Clustal W (http://www.ebi.ac.uk/clustalw/).

2.4 Expression, Purification, and Analysis of Protein

2.4.1 Pilot Expression of the Recombinant Protein

The full-length cDNA of CK-2 and CK-2.1 had already been cloned into pRSET A vector using *Bam*HI and *Hind*III restriction sites and transformed into BL21(DE3)pLysS strain of *E. coli* (previous student). Glycerol stocks prepared by this previous student were used to plate clones on LB plate containing ampicillin (amp) and chloramphenicol (chl) with the final concentration of 100 µg/ml and 40µg/ml respectively. For the chemokine domain and mucin stalk, after checking the sequence integrity, the pRSET A plasmid containing the insert was used to transform BL21(DE3)pLysS strain of *E. coli*.

Pilot induction was also carried out with BL21(DE3)pLysS (Novagen, WI, USA) cells. Briefly, 5 ml LB was inoculated with a single colony from the streak plates made from the glycerol stock and grown at 37^0C for over night; the media contained amp and chl with the final concentration of 100 µg/ml and 40µg/ml respectively. Next day, over night culture was used to inoculate 50 ml LB with amp and chl to an OD_{600} of 0.1; the overnight culture was spun down (4000rpm for 1minute) and re-suspended in fresh LB media. Furthermore, the overnight culture was used to make glycerol stocks (300µl of 50% glycerol and 700µl of culture). The culture was grown at 37^0C until an OD_{600} of 0.4-0.6. A 1 ml cell sample was taken before induction with isopropyl β-D thiogalactoside (IPTG) (Promega, WI, USA) as a non-induced sample. Furthermore, samples were taken 1hour (hr) interval for 5 hr. A 100µl Laemmli sample buffer (62.5 mM Tris-HCL pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.5% bromophenol blue) was added to each sample and mixed by pipetting up and down. The samples were then stored at -20^0C.

2.4.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The samples from the pilot expression study were boiled for 5 min and protein bands were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The 12% resolving gel was made with Acrylamide-Bis (30% T, 2.67% C) (Sigma-Aldrich, ON, Canada), 1.5 M Tris-HCL (pH 8.8), 10% SDS (Fisher Scientific), 10% Ammonium persulphate, and 0.05% TEMED (Sigma-Aldrich, ON, Canada). The 4% stacking gel was made of using above all, but the Tris-HCL buffer was 0.5 M with pH 6.8. Mini-Protein® II gel apparatus (Bio-Rad, CA, USA) was used to run the gels followed by the staining with Coomassie blue G-250 (J. T. Chemical Co, NJ, USA). Furthermore,

similar gels were transferred directly to nitrocellulose membranes in order to detect the bands with specific antibodies.

2.4.3 Western Blotting and Immunodetection

Following SDS-PAGE the proteins were transferred onto a nitrocellulose membrane for binding irreversibly. Briefly, the protein gel was placed on the top of a filter paper (FischerBrand, PA, USA). The nitrocellulose membrane (Pall Corporation, MI, USA) was placed directly onto the gel, and another filter paper was placed on the membrane. A test-tube was rolled over the filter paper to get rid of the bubbles. The whole sandwich was placed in between two Scotch-Brite pads, and then placed into the Trans-blot cell (Bio-Rad, CA, USA) with the nitrocellulose filter on the anode side and transferred at 33 V overnight. Next day, the membrane was stained with Ponceau S (0.1% Ponceau S and 5% glacial acetic acid) for 30 sec. The reaction was stopped with H_20. After scanning, the blot was blocked with 4% skim milk in T-TBS buffer (0.14 M NaCl, 2.7 mM KCL, 25 mM Tris, 0.5% Tween 20, pH 8) for 1 hr. The membrane was washed with T-TBS for 5 min and probed with mouse anti-express (Invitrogen, ON, Canada) at a final dilution to 1:5000 for 1hr. After 1 hr, the membrane was washed with T-TBS 3 times. Finally, the secondary antibody was added at 1:30,000 dilution and probed for another 1 hr. The secondary antibody was anti-mouse IgG (whole) alkaline phosphatase conjugate (Sigma, ON, Canada). Before detection the membrane was washed again with T-TBS 3 times and then equilibrated with alkaline phosphate (AP) buffer (0.1 M Tris, 0.1 M NaCl, 0.1 M $MgCl_2$ pH 9.5) for 5 min. To detect the signal, a substrate containing 0.33mg/ml nitro blue tetrazolium (NBT), 0.165 mg/ml bromochloroindolyl phosphate (BCIP) was prepared in AP buffer. The membrane was incubated for 15-30 min in the substrate until a purple signal developed. The reaction was then stopped by the addition of deionized water.

2.4.4 Bulk Expression of Recombinant Protein

Fifty ml LB medium was inoculated with a single colony from a streak of the glycerol stock made during the pilot expression. After overnight incubation, all 50ml culture was centrifuged at 4000 rpm for 1 min and re-suspended in fresh LB, which was then used to inoculate 500ml LB broth. The flask was placed in the environment-controlled Gyrotary G10 shaker (New Brunswick Scientific Co., NJ, USA) at 37^0C. The cells were grown until the OD_{600} was 0.4-0.6 and then induced with IPTG at a final concentration of 1mM. Following induction, the cells were grown for another 5 hrs to the point of highest yield as shown in the pilot expression study (section 2.4.1). The cells were harvested by

centrifugation at 10,000rpm for 10 min using the GSA rotor and Sorvall RC5B centrifuge (DuPont, USA).

2.4.5 Purification of the Recombinant Protein

The pellet from the 500ml culture was weighed. 10 ml binding buffer (100mM NaH_2PO_4, 10mM Tris, 8M urea) per 3 gm of pellet was added and homogenized using a dounce homogenizer (Kontes Glass, NJ, USA). After homogenization, another 40ml binding buffer was added to the cell lysate and mixed at RT using an orbital rotator (Adam Nutator, NJ, USA) overnight. Following incubation, the cell lysate was centrifuged at 10,000rpm for 30 min in order to remove bacterial cellular debris. The supernatant was collected and purified through Ni-affinity chromatography under denaturing conditions (8M urea). About 2ml Ni-Nitrilotriacetic acid (NTA) resin (Qiagen, ON, Canada) was washed with water and binding buffer, added to 15ml cell lysate and incubated for more than 2hrs at RT. Recombinant CK-2 (rCK-2) was purified via nickel affinity chromatography. After incubation, the lysate was loaded onto a 25ml column and washed with binding buffer equilibrated to pH 8.0, pH6.3, and pH 5.9. Finally rCK-2 was eluted at pH 4.5 and was stored at 4^0C.

2.4.6 Quantification of Protein

The Bradford Assay (Sambrook *et. al.,* 1989) was used to determine the concentration of protein. At first, a standard curve was plotted using several known concentration of bovine serum albumin (BSA). BSA was dissolved in binding buffer to prepare samples with known concentration ranging from 0µg/ml – 1000µg/ml. A 96 well plate was used for the assay. The standards were mixed with Bradford reagent (10% CBB G-250, 85% phosphoric acid, and 5% ethanol) in 1:50 dilution. After 5min of incubation the plates were read at 595nm using the Versamax micro-plate reader (Molecular devices, CA, USA). Once the absorbance readings were recorded, a standard curve was plotted using the concentration on the x-axis and the absorbance on the y-axis. The program Excel provided an equation, which was then used to determine the concentration of unknown rCK-2 dissolved in binding buffer.

2.4.7 Concentrating the Diluted Protein

The pooled rCK-2 was very dilute; therefore, the protein needed to be concentrated. In order to do that, dialysis tubing (Fisherbrand, ON, Canada) with 3,500 molecular weight cut-off was used. The dialysis bag was boiled for 5 min and protein solution was placed inside the bag. Two endings were closed using the clips provided with the bag. Polyethylene glycol 8000 (PEG 8000) (Sigma-Aldrich,

ON, Canada) was sprinkled on the bottom and top of the dialysis bag, which was sitting on a petri-plate. The plate was left at room temperature until the appropriate volume was reached.

2.5 Development of Polyclonal Antibodies

2.5.1 Antigen Preparation, Immunization, and Serum Preparation

Recombinant CK-2 was dialysed in 1x PBS, concentrated down to 1mg/ml and stored at 4^0C. Dialysis was carried out in a stepwise manner. The protein samples were dialysed from 8 M urea to 4 M urea; then to 2 M urea, to 1 M urea, and finally to 1XPBS. The protein was allowed to dialyse in 4 M and 2 M urea for 3 hr at RT; however, the dialysis into 1 M urea was carried out at 4^0C overnight. Also, the final dialysis into 1XPBS was performed at 4^0C overnight. Two rabbits were anaesthetized 1 hr prior to each injection. Before each injection, a small volume of (~10ml) blood sample was collected to determine the titre of the antibody. The injections were given sub-cutaneously at four sites per animal. Both rabbits were injected with an initial dose of 0.5 mg rCK-2 with Freund's complete adjuvant (50:50). A boost using 0.5 mg rCK-2 with Freund's incomplete adjuvant (50:50) was given every three weeks. Rabbits were exsanguinated after 12 weeks and approximately 60 ml of serum was collected from each rabbit. Serum was also collected from each blood sample obtained from the rabbits prior to each injection. Blood samples were allowed to clot at room temperature for 3 hrs. The sample was refrigerated at (4^0C) overnight in order to shrink the clot. Following clotting, the blood samples were centrifuged at 3,000x g for 10 min at 4^0C. The supernatant was saved and later centrifuged again to remove all the blood cells. Sodium azide was added prior to storage at a final concentration of 0.02% in order to reduce the bacterial growth. Rabbit serum was then stored at -20^0C. The veterinary technician of the University of Waterloo, Biology Department, performed administration of the injection and exsanguination.

2.5.2 Enzyme Linked ImmunoSorbent Assay (ELISA)

In order to determine the titre of the rabbit anti-CK-2 antibody, ELISA was performed on the serum collected from each blood sample drawn prior to booster injections. Recombinant protein was diluted in coating buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, and 3 mM NaN_3, pH 9.6) at a final concentration of 10μg/ml and 100μl was placed in the wells of a ninety-six well plate (Evergreen Scientific, CA, USA) in triplicate. Keyhole Limpet Hemocyanin (KLH) (Calbiochem, CA, USA) was used as a positive control. Moreover, 1:1000 fetal calf serum (FCS) served as a negative control to

account for background correction. The plates were incubated for two hrs at 37^0C. Following incubation, the wells were washed with T-TBS 3 times, and blocked by adding 300µl of blocking solution (1% BSA in T-TBS) to each well and incubating for 1 hr at RT. Again, the wells were washed 3 times with T-TBS following the blocking step. The titre was measured at several different dilutions of the primary antibody (anti-CK-2), namely 1:100, 1:500, 1:1000, 1:5000, 1:10000, 1:50000, 1:100000. The control serum, anti-KLH, was used at 1:1000 dilution. The dilutions were prepared in the blocking solution and 100 µl of sera dilutions were added to the wells and incubated for 1 hr at RT. The wells were washed (3 times) and probed with the secondary antibody: anti-rabbit IgG whole molecule alkaline phosphate conjugate at a final dilution of 1:5000 for 1 hr. Following T-TBS wash, substrate was added into each well for detection. The substrate was Sigma Fast p-nitrophenyl phosphate (p-NPP) tablet set (Sigma- Aldrich, ON, Canada). The plates were incubated in dark for 30 min and then the reaction was stopped by the addition of 0.03 M NaOH. The absorbance was measured at 405nm using the Versamax microplate reader (Molecular Device, CA, USA). The background reading was subtracted from the actual readings and the average of the three absorbencies was used to plot bar graph.

2.5.3 Affinity Purification of the Antibody

The final serum was affinity purified using a sulfolink affinity column containing recombinant protein. At first, column was bound with ~7mg protein (rCK-2) in coupling buffer (50 mM Tris, 5 mM EDTA-Na, pH 8.5) and incubated for 15 min with continuous shaking and then kept at 4^0C overnight (this increases binding affinity). After washing with 1XPBS (~6ml) and wash buffer (~12ml) (1.0 M NaCl, 0.05% NaN_3) the column was blocked with L-cysteine (2 ml of 8mg/ml in coupling buffer) and then crude antibody lysate was added to the column and incubated for 15 min at RT and the overnight at 4^0C. After overnight incubation the column was washed with sample buffer (~25ml) (0.1 M sodium phosphate, 5 mM EDTA-Na, pH 6) and then the purified antibody was eluted using 100mM glycine (pH 2.5). The reactivity of the purified antibody was detected using Western blot analysis as described in section 2.4.3.

2.5.4 Cross-reactivity of Anti-CK-2 Antibody

In order to determine whether the newly produced antiCK-2 antibody crossreacted with CK-2.1, Western blotting was carried out using three recombinant proteins: CK-2, CK-2.1, and beta-2-microglobulin (β_2M). Equal amounts of each recombinant protein were separated on a 15% SDS-

PAGE gel and transferred onto nitrocellulose membrane. Two individual blots were probed with two different kinds of antibodies. One blot was probed with purified anti-CK-2 antibody at a final dilution of 1:50 and the second blot was probed with purified anti-CK-2 blocked with recombinant β_2M in order to block the reactivity against his-tag. Blocking was achieved by adding about 3mg rβ_2M to 15ml of 4% milk solution containing anti-CK-2 antibody at 1:50 dilution, and incubating for 1 hr at RT. The antibody-protein solution was mixed using an orbital rotator.

2.5.5 Affinity Purification of Anti-CK-2 Antibody using Sulfolink Column Containing Mucin Stalk Protein

The anti-CK-2 antibody chemokine reactivity was affinity purified using a sulfolink column with the mucin stalk protein bound to it. The procedure was similar to the one described in section 2.5.3. The anti-CK-2 antibody was added to the column and incubated overnight. Following incubation, the flow-through was collected. The antibody collected in the flow through should only be reactive toward the CK2 chemokine domain. However, to ensure this, this antibody was further affinity purified using another column containing rCK-2 (section 2.4.3).

2.6 Transcript Expression in Primary Cultured Head Kidney (HK) and Peripheral Blood Leukocytes (PBL)

Several fish with various CK-2 allelic compositions (some with only CK-2, some with only CK-2.1, and some with both alleles) were chosen for the primary culture of HK and PBL.

2.6.1 Isolation of Head Kidney Leukocytes

A healthy rainbow trout was euthanized with MS-222 (0.01%). After removing all the blood through caudal vein the head kidney was taken out and placed into 20 ml of RPMI-1640-HAH (25 mM Hepes, 10000U/ml Pen, 10mg/ml Strep, 200 units/ml Heparin sodium salt) in a small petri plate on ice. The tissue was cut into smaller pieces and minced between two frosted glass plates in order to release the cells. The liquid containing the cells was transferred into a tube and centrifuged at 500rpm for 5 min. The pellet was washed two times with RPMI-1640-HA (0.025M Hepes, 10000U/ml Pen, 10mg/ml Strep). Around $1x10^7$ cells per plate were cultured in RPMI-1640-HA solution for 24 hrs at RT. The experimental plates were treated with 10μg/ml phytohemagglutinin (PHA) while the control was treated with saline (0.85% NaCl).

2.6.2 Isolation of Peripheral Blood Leukocytes

In order to isolate peripheral blood leukocytes, the blood was drawn from the caudal vein of rainbow trout. Prior to blood collection the syringe was filled with about 500µl heparin (200units/ml in 0.85% NaCl solution) (Sigma-Aldrich, ON, Canada) to prevent blood clotting. Once collected, the blood sample was centrifuged at 200x g for 10 min in swinging bucket rotor. After centrifugation, the buffy coat on the top of the red blood cell was removed and mixed with 45 ml of collection media (L-15, 10 IU heparin/ml, 2%FBS, 100U/ml Pen, 100µg/ml Strep). The mixture was split into six fractions and each was carefully layered on the top of 3 ml histopaque 1077 (Sigma-Aldrich, ON, Canada). The tubes were centrifuged for 30 min at 400x g. The PBLs were settled into the interface between the layer of histopaque and L-15. The PBLs were collected from this interface and pelleted by adding equal volume of collection media and centrifuging at 400x g for 5 min. The pellet was washed with 1 ml 1X PBS and resuspended in growth media (L-15, 20% FBS, 100U/ml Pen-Strep). The cell number was counted using a hemocytometer (Hausser Scientific, PA, USA). Briefly, a small volume of cells was diluted 5 times in L-15 and then an equal volume of 0.04% Trypan blue was added to that in order to stain the cells. Ten microliters of that cell suspension was placed on a hemocytometer to count the cells. Around $1x10^7$ cells/well were cultured in a six well culture plate for 24 hrs at RT. The experimental cells were treated with 10µg/ml PHA and the controls were treated with saline (0.85% NaCl).

2.6.3 RNA Extraction

After 24 hr incubation both PBLs and HKLs were collected with sterile cell scraper and centrifuged at 4000 rpm for 5 min. The pellet of cells was homogenized in 1ml of Trizol reagent (Life technologies, MA, USA) Following homogenization using a hand held electric pestle the samples were passed though 23 gauge needle few times to shear genomic DNA and then 200µl chloroform was added to the mixture and vortexed vigorously to mix properly. After incubating 5 min at RT the tubes were centrifuged at 12,000x g for 15 min. The transparent top layer was removed and placed into a new centrifuge tube and to that 500µl isopropyl alcohol was added. The tubes were inverted few times to mix and then incubated at RT for 10 min. Following incubation, the tubes were centrifuged at 12,000xg for 10 min. The RNA pellet was washed by resuspending in 1ml 75% ethanol made in diethyl pyrocarbonate (DEPC) water, and then centrifuging at 7500xg for 5 min. The pellet was air dried and resuspended in DEPC water. The concentration of RNA was determined by measuring the absorbance at 260nm using a UV-160 Spectrophotometer. The RNA samples were stored at -80^0C.

2.6.4 Reverse Transcription-Polymerase Chain Reaction (RT- PCR)

The RNA sample was transcribed into cDNA in order to perform PCR. An RT-PCR kit (MBI Fermentas, ON, Canada) was used to synthesise cDNA from RNA as per the manufacturers protocol, followed by PCR using forward primer CK-2 gDIG sense: 5'GCAGAAAAGCTGGTGTCGTG (Tm 57^0C) and reverse CK-2 antisense 5'GGAAGGTACGGATGGAGAAG (Tm 57^0C). Also, as an internal control PCR was performed using the primers for elongation factor alpha (EF1α). The primers for EF1α were, EF1α sense 5'-GAGTGAGCGCACAGTAACAC (Tm 57^0C), EF1α antisense 5'-AAAGAGCCCTTGCCCATCTC (Tm 57^0C). The template was diluted into 1:10 for EF1-α but used undiluted for the gene of interest. The thermal cycler was programmed as follows: 30 sec at 95^0C; 30 sec at Tm (57^0C for all the primers); 1 min at 72^0C; and 5 min at 72^0C. The second and third steps were repeated for 30cycles. The RT-PCR products were run on 1.5% agarose gel, stained with EtBr, and visualized with a UV transilluminator and EtBr filter.

2.7 *In vitro* study using cell lines

2.7.1 PCR on genomic DNA

All the cell lines were provided by Dr Niels Bols. Several rainbow trout cell line were used including, RTS11, RTG2, and RTS34ST. A PCR on the genomic DNA (provided by Bols lab) was performed to check the presence of CK-2 and CK-2.1 in these three cell lines. The primers were as follows: forward CK2 gDIG sense and reverse CK2 antisense (shown in section 2.6.4). Cycling conditions were the same as described in section 2.6.4.

2.7.2 CK2 and CK2.1 Expression in at Cells Line at the Transcript Level

Around $1x10^7$ cells were plated in each well of six well plates. The cells were suspended in 3 ml of L-15 media (Sigma, On, Canada) and incubated at 18^0C. A time course experiment was carried out by treating the cells with PHA at a final concentration of 10μg/ml and collecting samples after 0hr, 1hr, 2hr, 3hr, 4hr, and 24hr. After RNA extraction from each time point for each sample total RNA was used to perform RT-PCR (section 2.6.4). Two different culture conditions were used; one was a fetal bovine serum (Heat activated FBS) free environment and another was serum rich environment (15% FBS).

2.7.3 CK2 and CK2.1 Expression in the Cell Lines at the Protein Level

Around $2x10^7$ cells were plated in each well of six well plates. Again, the cells were treated with PHA (10μg/ml) and samples were collected after 0 hr, 1 hr, 2 hr, 3 hr, 4 hr, and 24 hr. The cells were centrifuged at 4,000rpm for 5min. Five hundred microliters of lysis buffer (1% nonidet P40 (NP-40), 150 mM NaCl, and 50 mM Tris-HCl pH 7.4 mixed with 1% protease inhibitor cocktail and 2mM PMSF) was added to the cells and they were incubated for 30 min on ice. Following incubation the cells were centrifuged for 15,000rpm for 15 min at 4^0C. The supernatant was collected and stored at -20^0C.

The protein sample was quantified by Bradford assay (section 2.4.6). In order to reduce the effect of NP-40, the protein sample was diluted. Two microliters of protein sample was added to 80 μl H_20. The sample was further diluted 1:10. The Bradford assay was performed by using 20μl from the diluted protein sample mixed with 200μl of Bradford reagent. In addition, a standard was produced using serial dilutions of BSA. The standards were also diluted using 2μl in 80μl of H_20.

2.7.4 SDS-PAGE and Western blot analysis

One hundred micrograms of protein was mixed with 5X Laemmli sample buffer and boiled for 5min. The samples were run on a 15% acrylamide gel and transferred overnight as described earlier (section 2.2.2 and 2.2.3). The blot was probed with a 1:50 dilution of anti-CK2 antibody and 1:1000 diluted secondary antibody (anti-rabbit HRP conjugated). The blots were detected using ECL plus Western blotting detection reagent (Amersham Biosciences). The blot was incubated in ECL plus reagent (1 ml of reagent A and 25μl of reagent B) for 5 min. Fluorchem 8000 was used to visualize the signals. The blot was exposed for 10min at the chemiluminesence setting.

2.8 *In Vivo* Study Using Rainbow Trout

2.8.1 PHA Injection Study

This study included rainbow trout containing a mixture of both CK-2 and CK-2.1 alleles. Therefore, several rainbow trout were genotyped (blood samples were collected and PCR was performed) to detect the allelic organization. Initially, two rainbow trout were given an intravenous injection of PHA at a final concentration of 50μg/250g of fish, and sacrificed after 24 hr as a pilot study. However, later on a total of 8 healthy rainbow trout were used for this injection study. Two fish

served as negative control and were not injected with anything. The other six fish were injected with PHA at a final concentration of 50μg/200g fish. Prior to injection the fish were anaesthetized with 0.01% MS-222. Two fish were sacrificed at each time point: 4 hr, 8 hr, and 24 hr. Following dissection, HK, Liver, PBL, Brain, Spleen, Intestine, Muscle, and Heart were collected into microcentrifuge tubes and immediately flash frozen in liquid nitrogen.

2.8.2 RNA extraction

All the tissue samples were kept on dry ice before a small portion was removed for RNA extraction. The rest of the tissue samples were stored for the protein isolation. About 50-100mg of each tissue was collected into microcentrifuge tubes containing 0.5ml Trizol. The rest of the RNA extraction procedure was similar to the one described in section 2.6.3. Again, the RNA samples were used to perform RT-PCR as described in section 2.6.4.

2.8.3 SDS-PAGE and Western Blot Analysis

The SDS-PAGE and Western blot were performed as mentioned in section 2.7.4.

2.9 Chemotaxis Assay

2.9.1 Isolation of PBLs

Rainbow trout were anaesthetized with 0.01% MS 222, and 3ml blood was drawn from the caudal sinus with 23G needle. The PBLs were isolated as discussed in section 2.6.2. For this purpose PBLs were washed with 10ml PBS before use in the chemotaxis assay. Cells were resuspended in L-15 at a final concentration of 1×10^7 cells/ml.

2.9.2 Preparation of RTS-11 Cells

RTS-11 cell were provided by Dr. Niels Bols. Cells were diluted at a final concentration of $1x10^7$ cells/ml in L-15 media without any FBS or antibiotics.

2.9.3 Preparation of Zymosan Activated Serum

One hundred milligrams zymosan (Sigma-Aldrich, ON, Canada) was suspended in 10ml of 0.85% NaCl solution (saline). The suspension was heated in boiling water for 30 min followed by centrifugation at 3,000rpm for 10 min. at RT. The supernatant was discarded and the pellet was

suspended in 10 ml of saline and vortexed, again the mixture was centrifuged at 3,000rpm for 10 min RT. The above step was followed once more and after centrifugation the pellet was suspended in 10ml saline and 10% NaN_3 at a final concentration of 0.02%. The final product was stored at 4^0C. The fish serum was prepared the same way as the rabbit serum as described in section 2.5.1. In order to activate the serum with zymosan, one volume of zymosan suspension was placed in to a microcentrifuge tube and centrifuged at 3,000rpm for 10 min at 4^0C. A 10 volume of serum was added to the pellet and suspended by pipetting and incubated at RT for 30min with 10min interval of mixing by inverting. The serum was then centrifuged at 3,000 rpm for 10 min. at 4^0C. The supernatant was stored at -80^0C.

2.9.4 Preparation of Proteins

Recombinant β_2M was also produced for this assay. A glycerol stock provided by Stephen Kales, was used to produce about 10 mg of protein. Both CK-2 and β_2M were dialyzed in 1xPBS (stepwise dialysis from 8 M urea to 4 M urea, 4 M urea to 2 M urea, 2 M urea to 1 M urea, 1 M urea to PBS). The whole dialysis process was carried out at 4^0C. Several different concentrations were used including 10 μg/ml, 1 μg/ml, 500 ng/ml, and 100 ng/ml of protein in 1xPBS.

2.9.5 Blocking Experiment

Two different aliquots of PBLS were blocked with CK-2 (500 ng/ml) and pertussis toxin (100 ng/ml), respectively. Also, CK-2 (500 ng/ml) was blocked with anti-CK-2 antibody. All these three samples were incubated on orbital shaker at RT for 1 hr. Following incubation, the cells were washed twice with 1xPBS (resuspended in PBS and centrifuging at 4,000rpm for 3min) and finally resuspended in L-15 at a final concentration of $1x10^7$ cells/ ml for use in the chemotaxis assay. The blocked CK-2 was used directly.

2.9.6 Chemotaxis Assay

A 48-well micro chemotaxis assembly (Neuro Probe, MA, USA) was used to perform chemotaxis assays. In short, different concentrations (10 μg/ml, 1 μg/ml, 500 ng/ml, 100 ng/ml) of several proteins (CK-2, CK-2.1, β_2M, mucin, ZAS, PBS) were loaded into different wells in the bottom chamber. On the top of the lower chamber a polyvinylpyrrolidone (PVP) free polycarbonate membrane with a pore size of 5μm was placed. The upper chamber was filled with 45μl of cell suspension of 1 x 10^7 cells/ml in L-15 media. The assembly was incubated for 1 hr at RT. RTS-11

cells were incubated for 2 hr since these cells are much bigger than PBLs. Following incubation the filter was removed; the top surface was scraped, and washed 3 times with PBS. The filter was then fixed in methanol for 1min and stained with 1% Giemsa (dissolved in 100% Ethanol) for 1 hr. After staining the filter was washed 2 times with deionized water and mounted on a microscope slide after fixing with Permount (Fisher Scientific, PA, USA). The bottom side of the filter was used to count the migrated cells. A total five different fields per well were counted using a light microscope at 400x magnification. In order to calculate the chemotactic index, the number of cells that migrated in response to chemokines was divided by the number of cells that spontaneously migrated (toward negative control, PBS).

2.10 Densitometry Analysis of RT-PCR

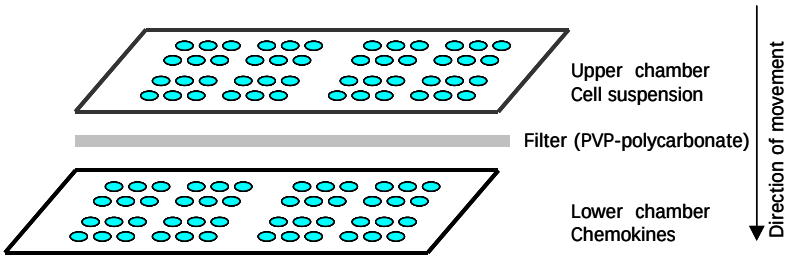
RT-PCR gels were analyzed using NIH Imager v. 1.63 in order to compare the band intensity between the gene of interest (CK-2 and CK-2.1) and internal control (EF1-α). Initially, the background reading was taken by creating a very small area spanning the entire height of the image. Using the same area, the signals for the gene of interest and the internal control were detected. The signal peaks were represented by pixels. The peaks for the internal control and gene of interest were superimposed over the background reading, and the area of the peak was measured. A relative change in expression was calculated by comparing the band intensity between the internal control and gene of interest bands.

2.11 Statistical Analysis

The average of individual experiments was taken and plotted a bar graph using Microsoft Excel. The standard deviations and the error bars were also calculated and added on the graph. Furthermore, the data (the ratios in case of RT-PCR and the total number of migrating cells in case of chemotaxis assay) was transformed by taking the natural logarithm (ln) in order to compensate for the variations in different factors that might not have been consistent throughout the experiment (temperature, the handling of cells, etc.). To know the statistical significance, statistical analysis was conducted using the statistics program SPSS v. 12; a one-way ANOVA was performed on the transformed data followed by Fischer's Least Significant Difference (LSD) post hoc test. Raw data can be found in the Appendix B.

Figure 2.3: A 48-well micro chemotaxis assembly. The bottom chamber was loaded with proteins (CK-2 and other controls). On the top of the lower chamber, a PVP free filter with a pore size of 5μm was placed. The upper chamber was filled with 45μl of cell suspension containing 1 x 10^7 cells/ml. The assembly was incubated for 1 hr at RT and cells were expected to migrate toward the chemokines. **(A)** Photograph of the chemotaxis assembly (the picture was adopted from http://www.neuroprobe.com/). **(B)** A schematic diagram showing the set up. The bottom chamber contains the chemoattractant and the top chamber contains the cell (PBLs, RTS11). In between the two chambers, a filter is placed though which the cells are migrated and attached at the bottom surface of it.

A


Upper chamber
Cell suspension
Filter (PVP-polycarbonate)
Lower chamber
Chemokines
Direction of movement

B

Chapter 3
Results

3.1 Genomic Expression of CK-2 and CK-2.1

Blood samples were collected from several fish. After genomic isolation, the DNA was subjected to PCR using different sets of primers flanking the extra mucin stalk in CK-2.1. Different primer sets produced different sizes of products ranging from 500bp to 700bp. Figure 3.1 shows results from the PCR using one set of primers. The gel image shows the presence of two distinct bands with the size of 600bp and 700bp. However, not all the fish tested contain both bands. Among ten different rainbow trout, only 3 fish have CK-2; 3 have CK-2.1; and rests of them have both CK-2 and CK-2.1. Therefore, it can be concluded that CK-2.1 is an allele of CK-2. If CK-2.1 were a separate gene it would have been present in all ten fish. Moreover, over time more fish were genotyped for various experiments. A total of 55 fish have been genotyped. Among them, 20% had only CK-2, 36% had CK-2.1, and 44% had both CK-2 and CK-2.1. Therefore, many of the fish have both alleles. If we consider the Hardy-Weinberg law that gives us an equation of $p^2+2pq+q^2=1$, we can see that the results from our experiment gives a value of 1: $(0.42)^2+2(0.42)(0.58)+(0.58)^2=1$.

A Southern blot analysis (figure 3.2) was also performed to detect the pattern in both homozygous and heterozygous fish. The genomic DNA from rainbow trout was digested with the RE (*Hind*III, *Eco*RI, and *Pst*I) and subjected to Southern blot analysis. Only one band is present in all three rainbow trout DNA digested with *Eco*RI. *Pst*I creates two bright bands and four faint bands in DNA from the fish having both CK-2 and CK-2.1, while fish with CK-2 shows three bands and fish with CK-2.1 shows four bands. Some of the bands from the homozygous fish match with the heterozygous fish. Moreover, digestion with *Hind*III makes two bright bands in fish with CK-2 and another fish with both CK-2 and CK-2.1 and only one band in fish with CK-2.1 alone. The lower band in CK-2 & CK-2.1 fish matches with the one present in CK-2.1 fish. Also, if we add the sizes of two bands (~10kb and 8kb) present in CK-2 fish we can probably find the same size band (the upper band, ~18kb) seen in the fish with both alleles. The two bands seen in the CK-2 fish probably result from the longer intron sequence; therefore, having more probability of containing a cut site for restriction enzymes. Another possible explanation could be the presence of different sizes flanking regions in individual fish.

Figure 3.1: PCR of genomic DNA from rainbow trout. About 100µl blood was drawn from each fish. The genomic DNA from each sample was used to perform PCR with LA taq (Takara, Japan). The primer used here flanked the extra mucin domain present in CK2.1. The PCR products were run on 1.5% agarose gel, stained with EtBr and visualized with UV light. The 700bp band represents CK2.1 whereas the 600bp represents CK2. Fish 1, 5, 6, and 8 have both CK2 and CK2.1, whereas fish 2, 3, and 4 have only CK2 and fish 7, 9, 10 have only CK2.1.

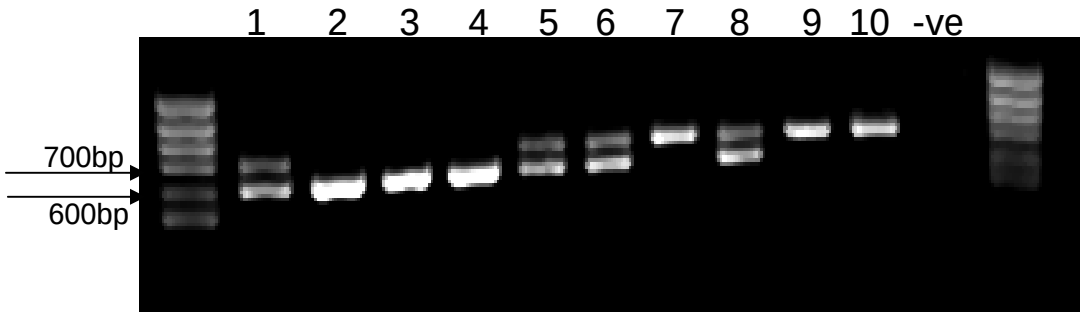
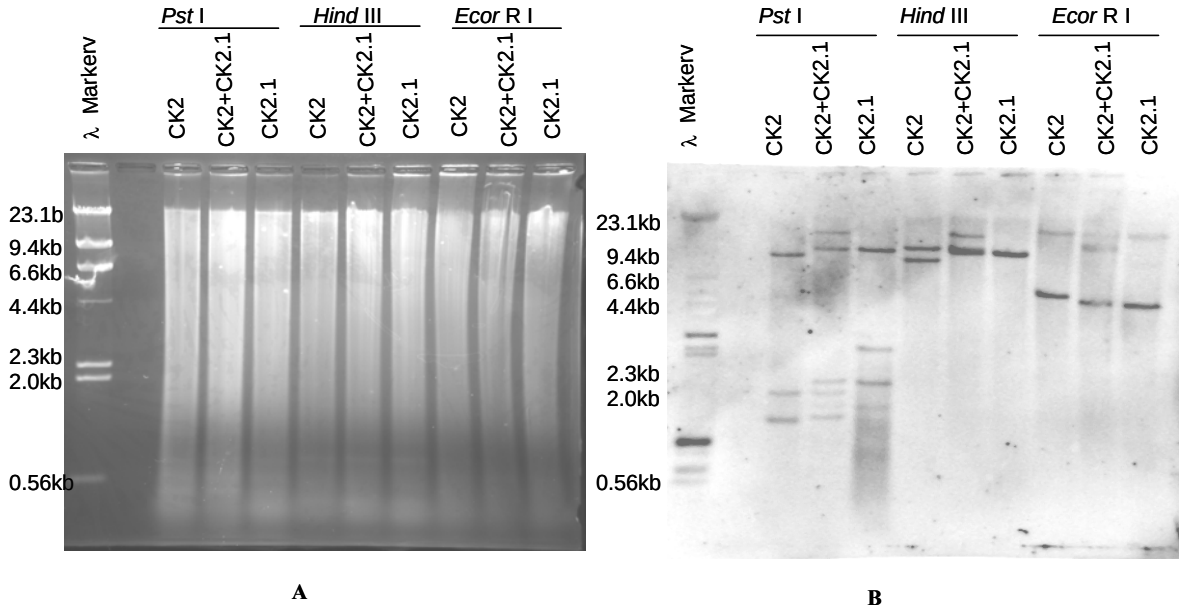

1
2
3
4
5
6
7
8
9
10
-ve
700bp
600bp

Figure 3.2: Southern blot analysis of genomic DNA from rainbow trout. The genomic DNA was extracted from three rainbow trout (homozygous and heterozygous) and subjected to RE digest. After the digest, the products were run on 1% agarose gel, DNA was denatured, and transferred onto a nylon membrane. Following transfer, the blot was hybridized with DIG labeled probe and the signal was detected by probing the membrane with alkaline-phosphate conjugated anti-DIG antibody and incubating with CDP star. In the end, the membrane was developed using Fluorchem 8000 (Alpha Innotech). The membrane was exposed for 20min using chemiluminesence filter at medium sensitivity. (A) The agarose gel photograph showing the complete digestion of the genomic DNA. (B) The nylon membrane representing the signal after chemiluminesence detection.


Pst I
Hind III
Ecor R I
λ Markerv
CK2
CK2+CK2.1
CK2.1
23.1b
9.4kb
6.6kb
4.4kb
2.3kb
2.0kb
0.56kb
23.1kb
9.4kb
6.6kb
4.4kb
2.3kb
2.0kb
0.56kb

A **B**

Hence, the southern results are also consistent with the genomic PCR indicating CK-2.1 is an allele but not a separate gene. However, in general, fish are too genetically diverse to see any consistent patterns.

3.2 Expression and Purification of Proteins

All four proteins were cloned into pRSET A vector and transformed into BL21(DE3)pLysS strain of *Escherichia coli* . Following the pilot induction assay, protein was bulk expressed and afterwards purified via nickel affinity chromatography. Figure 3.3 represents the different elution steps during the purification of rCK-2 and rCK-2.1, and figure 3.4 shows the similar kinds of results for rChemokine and rMucin. The recombinant protein was bound to the nickel resin via 6x histidine tag. Other bacterial proteins that lacked the tag passed though the column when washed with lysis buffer with different pHs. The column with lysate bound to it was washed with lysis buffer at pH 8.0, 6.3, and 5.9. Lastly, all the recombinant proteins were eluted with pH 4.5. However, some of the recombinant proteins appeared to be eluted at pH 6.3 or pH 5.9. This could be due to the saturation of nickel resin, which left some protein unbound.

In order to determine the sizes of the proteins, samples from different washes were run on a 12-17% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was stained with Ponceau S and washed with T-TBST. Followed by washes the membrane was blocked with 4% milk solution; probed with primary (mouse anti-express antibody) and secondary antibody (anti-mouse AP-conjugated) and detected with the addition of substrate made of NBT and BCIP. From figure3.3 and figure 3.4, it is clear that all the recombinant proteins were eluted at pH 4.5. The approximate sizes of the four proteins are: rCK-2 is 30kDa (expected 21kDa), rCK-2.1 is 35kDa (expected 25kDa), rMucin is 17kDa (expected 10.4kDa), and rChemokine is 15kDa (expected 7.2kDa). The sizes are little higher than expected which could be due to the presence of proline and cysteine residues interfering with the migration of the proteins. In addition, presence of his-tag provided some extra mass (~3kDa). Sometimes a higher band is also visible which could be due the formation of multimers, possibly dimers.

3.3 Development and Purification of Anti-CK-2 Polyclonal Antibody

Polyclonal antibody was developed only against rCK-2. After the bulk induction, the rCK-2 was diluted in 1XPBS and injected into rabbits. Each rabbit was given an initial dose with 0.5mg rCK2

mixed with Freund's complete adjuvant followed by three subsequent injections with 0.5mg rCK2 mixed with Freund's incomplete adjuvant. Besides, ELISA was performed to check the titre of the antibody prior to each injection. Figure 3.5 shows the titre of the antibody raised in rabbit#1 (Please refer to Appendix C for the titre of the antibody raised in rabbit#2).

In order to perform ELISA, the plates were coated with 1 μg rCK-2 per well followed by blocking and probing with polyclonal antibody developed in rabbit. Several different dilution were used including, 1:100, 1:500, 1:1000, 1:5000, 1:10,000, 1:50,000, 1:100,000. After probing with secondary, the signal was detected with the addition of pNPP to each well. Figure 3.5 represents the titre of rabbit #1, which shows that after the final bleed, the titre is quite strong for even 1:100,000 dilution. Also, the graph represents the increasing pattern after each successive injection. With each successive boost the reactivity to each dilution increased except for the final bleed. Nevertheless, the rabbit was exsanguinated one week earlier than the scheduled time, which might be the reason for a lower reactivity than expected. However, the reactivity is higher for the higher dilutions (1:50000 and 1:100000). Overall, the serum was quite reactive to the recombinant protein. In the end, serum for rabbit#1 was used for the Western blots since it showed better reactivity than serum from rabbit#2.

The anti-CK-2 antibody was also purified via sulfolink column. The rCK-2 was bound with sulfolink resin and the crude serum was passed though the column. The column was washed to remove all the non-specific antibodies. Eventually, at a very low pH the antibody specific to CK2 was eluted. Again, a Western blot was performed to detect the reactivity of the purified antibody. The reactivity was at its peak when the dilution was 1:50. Figure 3.6 shows the results before and after purification of the antibody. The purified antibody represented a cleaner signal than the non-purified antibody.

Furthermore, the cross-reactivity of newly produced anti-CK-2 toward CK-2.1 was determined. This was done by blocking the anti-CK-2 antibody with rβ_2M and probing the blot containing three recombinant proteins, including CK-2, CK-2.1, and β_2M. When the blot was probed with purified anti-CK-2 antibody, signals were detected for all three proteins. This was due to the presence of his-tag in all the proteins. However, after blocking the anti-CK-2 with rβ_2M, it did not recognize β_2M anymore due to the removal of his-tag antibody, but it still recognized CK-2.1 (figure 3.6) suggesting the fact that anti-CK-2 antibody is able to recognize CK-2.1.

Figure 3.3: Purification of the recombinant CK2 and CK2.1 by nickel affinity chromatography. The bacterial lysate containing recombinant proteins was bound with Ni-NTA resin and washed with 8M-urea buffer of pH 8.0, 6.3, and 5.9. Both rCK2 and rCK-2.1 were eluted with the same buffer of pH 4.5. Samples from different elutions were run on a 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The primary antibody was added at a final dilution of 1:5000; the secondary antibody was added at a 1:30,000 dilution; and the blots were detected by adding a substrate made of NBT and BCIP. (A) Ponceau S staining of the nitrocellulose membrane containing samples from purification of CK-2. (B) The membrane was probed with anti-express^{TM} antibody, which confirms the presence of rCK2 (~30kDa band with his-tag). (C) Ponceau S staining of the membrane containing samples from purification of CK-2.1. (D) The membrane was probed with anti-express^{TM} antibody, which confirms the presence of CK-2.1 (~35kDa band with his-tag).

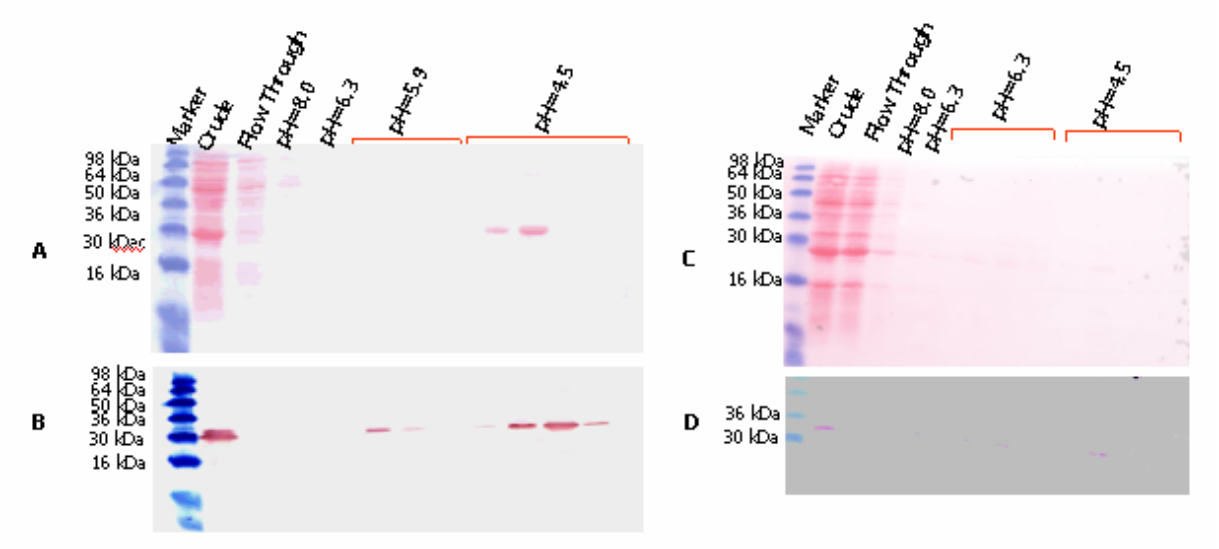

A
B
C
D
Marker
Crude
Flow Through
pH=8.0
pH=6.3
pH=5.9
pH=4.5
98 kDa
64 kDa
50 kDa
36 kDa
30 kDa
16 kDa

Figure 3.4: Purification of the mucin stalk protein and chemokine domain protein by nickel affinity chromatography. The bacterial lysate containing the recombinant proteins was bound with Ni-NTA resin and washed with 8M-urea buffer of pH 8.0, 6.3, and 5.9. Both chemokine domain and mucin stalk proteins were eluted with the same buffer of pH 4.5. Samples from different elution were run on a 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The primary antibody was added at a final dilution of 1:5000; the secondary antibody was added at a 1:30,000 dilution; and the blots were detected by adding a substrate made of NBT and BCIP. (A) Ponceau S staining of the membrane with nickel elutions. (B) The membrane was probed with anti-express^{TM} antibody, which confirms the presence of recombinant mucin stalk protein (~17kDa with a his-tag). (C) Ponceau S staining of the membrane representing nickel elutions. (D) The membrane was probed with anti-express^{TM} antibody, which confirms the presence of recombinant chemokine domain protein (~ 15kDa with a his-tag).

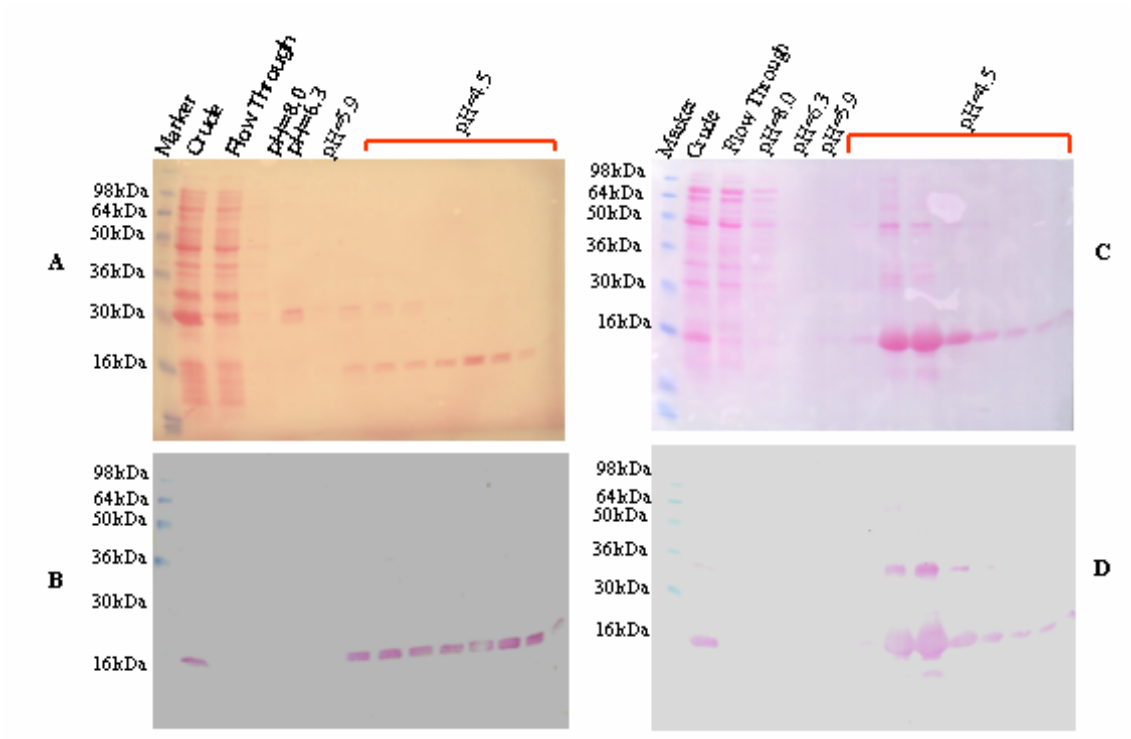

Marker
Crude
Flow Through
pH=8.0
pH=6.3
pH=5.9
pH=4.5
98kDa
64kDa
50kDa
36kDa
30kDa
16kDa
A
B
C
D

Figure 3.5: ELISA assay to determine the titre of the rabbit anti-CK-2. About 10ml rabbit blood was collected before each injection and used to perform ELISA. Serum was separated from erythrocytes by allowing the blood sample to coagulate. ELISA plates were coated with 1μg rCK-2. After blocking, the plates were probed with primary antibody (rabbit serum) with several dilutions. Following incubation, the secondary antibody (anti-rabbit AP-conjugated)) was added at a final dilution of 1:5000. Upon addition of the substrate, a colored product was formed, which was measured at 405nm. After each successive injection the titre increased gradually. The antibody quality was very efficient since it reacted against a very diluted rCK-2 (1:100,000).

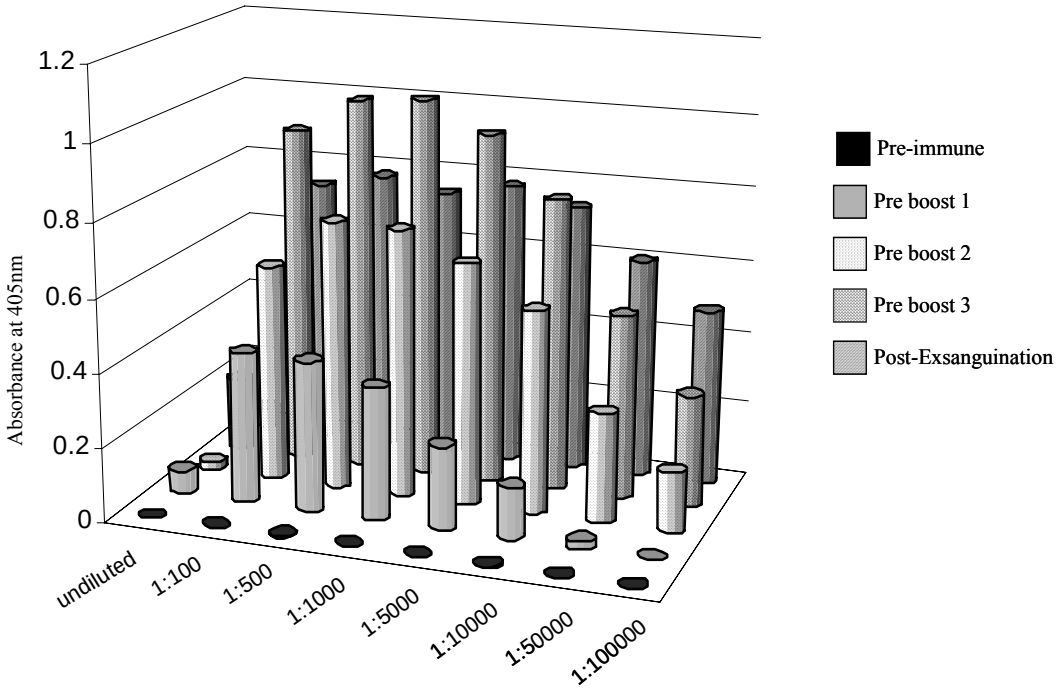
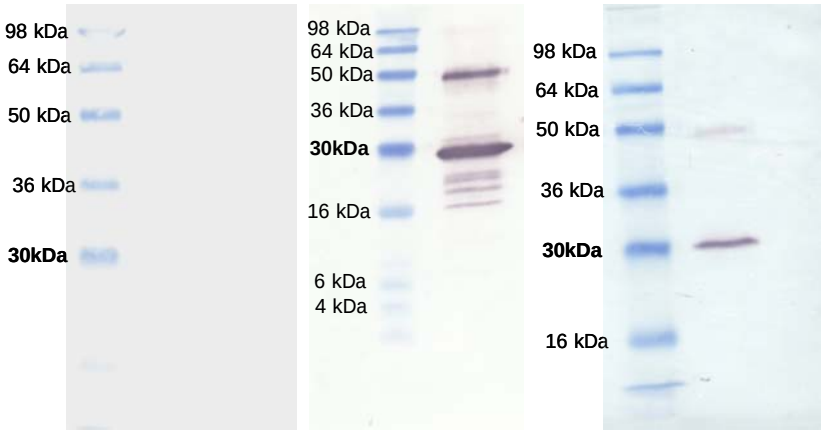
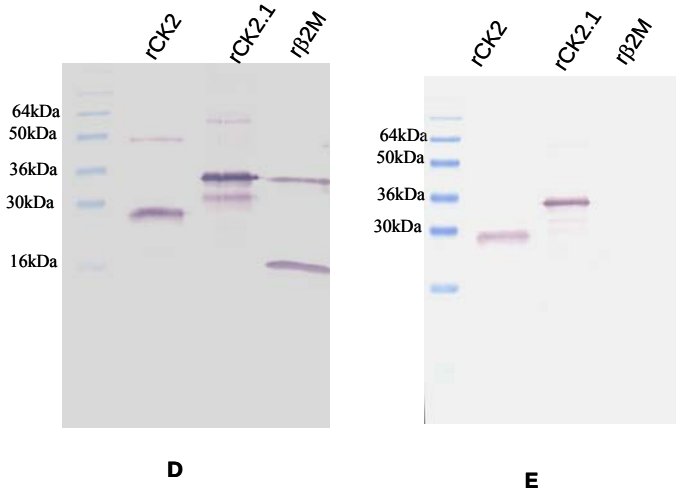

Absorbance at 405nm
1.2
1
0.8
0.6
0.4
0.2
0
undiluted
1:100
1:500
1:1000
1:5000
1:10000
1:50000
1:100000
Pre-immune
Pre boost 1
Pre boost 2
Pre boost 3
Post-Exsanguination

Figure 3.6: Western blot analysis to detect the reactivity of anti-CK-2 antibody. About 3μg rCK-2 was loaded to a 12-15% acrylamide gel and transferred to a nitrocellulose membrane. Later, the membrane was probed with primary antibody. (A) The membrane was probed with pre-immune serum (B) probed with crude anti-CK-2 at a 1:5000 dilution (C) probed with purified anti-CK-2 at a 1:50 dilution. After washing, all the membranes were probed with secondary antibody to a 1:30,000 dilution. The secondary antibody was anti-mouse IgG (whole) with alkaline phosphatase conjugate. The signals were detected by adding NBT and BCIP. (D) and (E) The cross-reactivity of anti-CK-2 toward CK-2.1. The anti-CK-2 antibody was used to probe two blots containing rCK-2, rCK-2.1, and rβ_2M. When the anti-CK-2 antibody is blocked with rβ_2M, (E) it did not recognize rβ_2M anymore. The reactivity against rβ_2M was due to the his-tag that was associated with all three recombinant proteins.


98 kDa
64 kDa
50 kDa
36 kDa
30kDa
A
98 kDa
64 kDa
50 kDa
36 kDa
30kDa
16 kDa
6 kDa
4 kDa
B
98 kDa
64 kDa
50 kDa
36 kDa
30kDa
16 kDa
C


rCK2
rCK2.1
rβ2M
64kDa
50kDa
36kDa
30kDa
16kDa
D
rCK2
rCK2.1
rβ2M
64kDa
50kDa
36kDa
30kDa
E

3.4 Expression of CK-2 *In Vitro* at the Transcript Level

3.4.1 Primary Culture of HKL and PBL

In order to detect *in vitro* expression of CK-2 and CK-2.1, the primary culture of HKL and PBL were used to examine the transcript level of CK-2 and CK-2.1. Rainbow trout containing three different allelic compositions were chosen. Therefore, both homozygous (for CK-2 and CK-2.1) and heterozygous fish were used in this purpose. After collecting the HKL and PBLs, the cells were plated and treated with PHA and 0.85% saline (control). Cells were harvested after 24hr. Total RNA was collected and subjected to cDNA synthesis followed by PCR with primers spanning introns, which would eliminate the possibility of genomic DNA contamination. RT-PCR, instead of northern blot, was performed on the total RNA due to the low yield of RNA. EF1-α primers were used to amplify this housekeeping gene; therefore, the expression of this gene should not change due to any stimulation. In order to provide better comparison, the PCR cycle was kept consistent for both gene of interest and internal control (30 cycles). The primers for the gene of interest were supposed to amplify a 331bp band for CK2 and a 456bp band for CK2.1. The primers specific to EF1-α were supposed to amplify a 250bp fragment. The agarose gel image in figure 3.7 represents the PCR products of expected bands for both the gene of interest and the internal control.

This experiment was repeated four times, but due to problems with RNA degradation only two repetitions were used for the analysis. A representative figure is shown here to show the transcript expression. As shown in figure 3.7, in HKL, the expression of both CK-2 and CK-2.1 is upregulated following stimulation. However, the heterozygous fish seemed to contain a lower initial level of CK-2.1 than CK-2. On the other hand, transcript expression shows a different pattern in PBLs with a very slight up-regulation after stimulation. A one-way ANOVA was performed to detect the statistical significance of the data. All the data were transformed into natural logarithm. The increase in transcript level in primary cultured PBLs did not show statistical significance ($p=0.118$). However, the overall ANOVA was significant for primary cultured HKLs ($p=0.016$).

3.4.2 Rainbow Trout Cell Lines RTS-11, RTG-2, and RTS34ST

Another *in vitro* experiment was designed to detect transcript expression using three different rainbow trout cell lines. An initial PCR was performed using the genomic DNA from all the cell lines in order to detect the presence of specific alleles in a specific cell line. PCR result with RTS-11 (figure 3.8)

showed that, it only contains CK-2. Knowing that, RTS-11 cells were cultured in six wells culture plate at a density of $1x10^7$ cells/well. Cells were treated with PHA at a final concentration of 10μg/ml. Also, one well served as a control, which was treated with saline (0.85% NaCl). Cells were collected 1hr, 2hr, 3hr, 4hr, 24hr following PHA stimulation. Again, RT-PCR was performed using primers specific to CK-2/CK-2.1 and EF1-α.

The PCR products from each set of primers were run on a 1.5% agarose gel. Densitometry analysis was also performed using the ratio of the bands. The agarose gel image in figure 3.8 does not show any change in transcript expression after stimulation. However, the bar graph represents that in FBS rich environment, the level of CK-2 is highest when there is no PHA. The expression of CK-2 decreases with time. The results are little different when there is no FBS in the media. The expression of CK-2 seems to be upregulated at 2hr and 4hr post stimulation and down-regulated 24hr post stimulation. The average from three different experiment was taken and used to plot bar graph. Statistical significance was conducted using a one-way ANOVA with a Fisher's post hoc test. The experiment with the presence of FBS showed no significance since it provided a p value of 0.726. However, the data from the experiment without the presence of FBS showed statistical significance ($p=0.046$). These data were transformed into natural logarithm.

The second cell line was RTG2, which is a rainbow trout fibroblast cell line. Again, the experimental conditions were similar to the ones used for RTS-11. Figure 3.9 reveals that RTG-2 has only CK-2.1 in its genomic DNA; however, the RT-PCR analysis does not show any transcript expression in any of the experimental conditions. The third cell line was RTS34ST. This was a stromal cell line. Like RTG2, RTS34S2 does not show any expression of CK-2 and CK-2.1, although the genomic DNA shows the presence of both CK-2 and CK-2.1 (figure 3.10). Due to cell death, the experiment without FBS was not repeated for RTS34ST cell line.

3.5 Expression of CK-2 *In Vitro* at the Protein Level

Only RTS-11 was used for the detection of CK-2 at the protein level. RTS-11 cells were cultured at a concentration of $2x10^7$cells/well in a six well plate. The experimental condition involved two conditions: one with the presence of FBS and one without FBS. The cells were stimulated with PHA (10μg/ml) and samples were collected after 1hr, 2hr, 3hr, 4hr, and 24hr (48hr and 72hr samples were also collected for the experiment with FBS).

Figure 3.7: Transcript expression of CK-2 and CK-2.1 in the primary culture of HKL and PBLs. About $1x10^7$ cells were plated in each well of six wells cell culture plates. One plate was used as a control and was treated with 0.85% NaCl (because the PHA was dissolved in saline) and another plate was treated with PHA and used as experimental. The plates were incubated for 24hrs at RT. RT-PCR was performed on the total RNA collected from the cells. (A) RT-PCR results for the HKLs (B) RT-PCR results for the PBLs. All the gels were run on a 1.5% agarose gel, stained with EtBr, and visualized under UV-light. As an internal control EF1-α was used. The bar graphs represent the average of ratios (the band intensity for gene of interest divided by the band intensity for internal control) derived from two independent experiments. Statistical significance was determined using a one-way ANOVA. The data were transformed into natural logarithm. No significance was observed in primary cultured PBLs ($p=0.118$). However, the overall ANOVA was significant for primary cultured HKLs ($p=0.016$). Raw data can be found in Appendix B. (*) Represents the significant change.

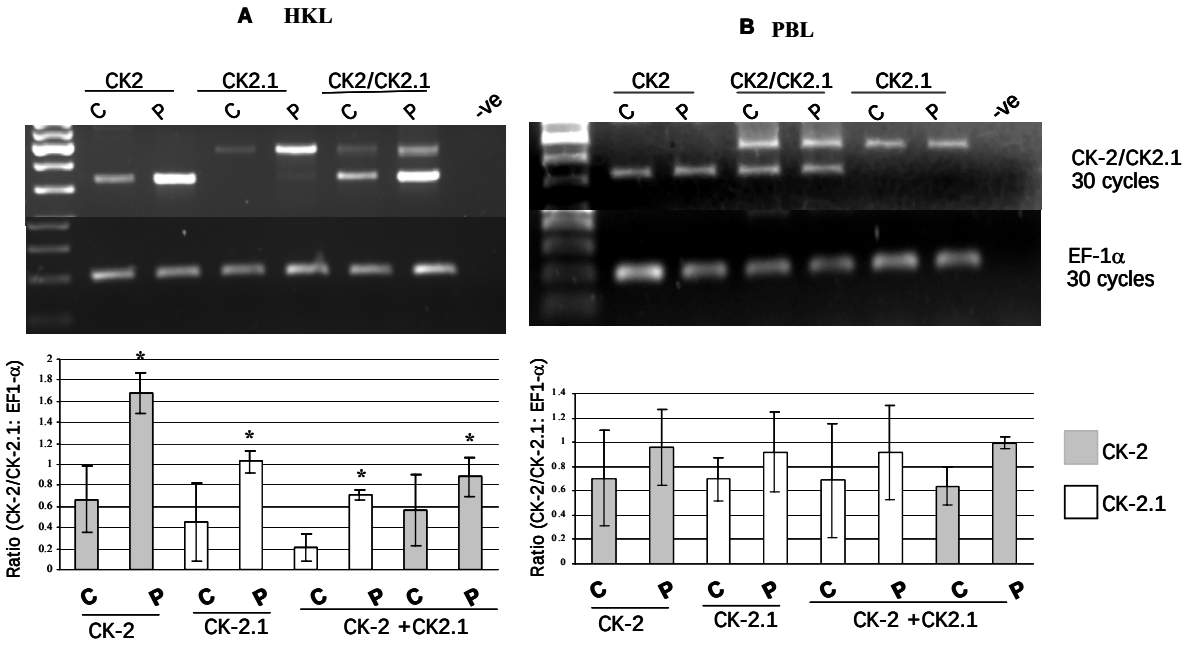

A HKL
B PBL
CK2
CK2.1
CK2/CK2.1
C
P
-ve
CK-2/CK2.1
30 cycles
EF-1α
30 cycles
Ratio (CK-2/CK-2.1: EF1-α)
CK-2
CK-2.1
CK-2 +CK2.1

Figure 3.8: RT-PCR with RNA samples collected from PHA treated RTS-11 cell line. (A) Genomic PCR to determine the allelic distribution of CK2 and CK2.1. The PCR products were run on a 1.5% agarose gel. Five hundred ng (0.5μg) of total RNA, isolated from the RTS11 at different times points (0hr, 1hr, 2hr, 3hr, 4hr, 24hr), was used to perform RT-PCR. Two sets of experiment were performed in the presence or absence of FBS. (B) RT-PCR with RNA from RTS11 grown in medium containing FBS (C) media lacking FBS. All the RT-PCR products were run on 1.5% agarose gel, stained with EtBr, and visualized under UV-light. The bar graphs show means and standard errors of three separate experiments. Statistical significance was conducted using a one-way ANOVA with a Fisher's post hoc test. The experiment with the presence of FBS showed no significance ($p=0.726$). However, the data from the experiment without the presence of FBS showed statistical significance ($p=0.046$). These data were transformed into natural logarithm. (*) Represents the significant change.

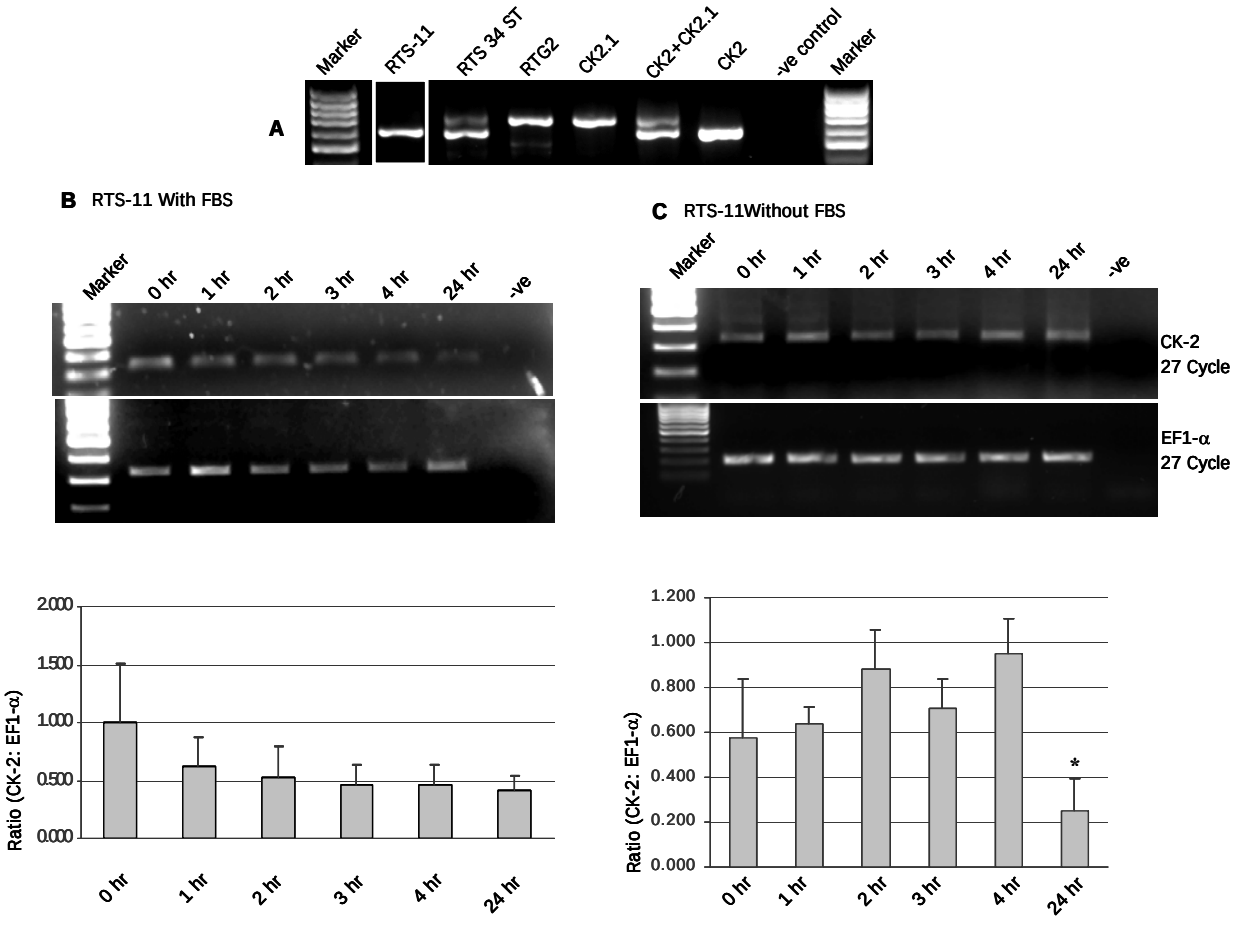
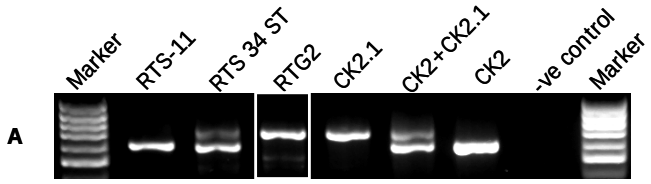
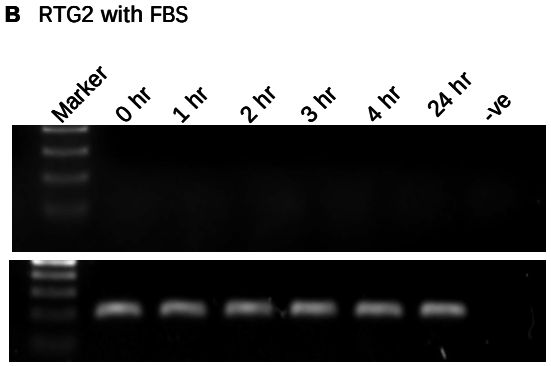

A
Marker
RTS-11
RTS 34 ST
RTG2
CK2.1
CK2+CK2.1
CK2
-ve control
Marker
B RTS-11 With FBS
Marker
0 hr
1 hr
2 hr
3 hr
4 hr
24 hr
-ve
Ratio (CK-2: EF1-α)
2.000
1.500
1.000
0.500
0.000
0 hr
1 hr
2 hr
3 hr
4 hr
24 hr
C RTS-11Without FBS
Marker
0 hr
1 hr
2 hr
3 hr
4 hr
24 hr
-ve
CK-2
27 Cycle
EF1-α
27 Cycle
Ratio (CK-2: EF1-α)
1.200
1.000
0.800
0.600
0.400
0.200
0.000
*
0 hr
1 hr
2 hr
3 hr
4 hr
24 hr

Figure 3.9: RT-PCR with RNA samples collected from PHA treated RTG2 cell line. (A) The genomic DNA was extracted and PCR was performed to check the allelic distribution of CK2 and CK2.1. The PCR products were run on a 1.5% agarose gel, stained with EtBr and visualized under UV-light. Five hundred ng (0.5μg) of total RNA was used to perform RT-PCR. Two sets of experiment were performed in the presence or absence of FBS. The top picture is for the gene of interest whereas the bottom picture is for the internal control EF1-α. (B) The RT-PCR results showing the transcript expression pattern. The experimental condition lacked FBS. (C) Similar experiment like B; only difference was that media contained 15% FBS.


A
Marker
RTS-11
RTS 34 ST
RTG2
CK2.1
CK2+CK2.1
CK2
-ve control
Marker


B RTG2 with FBS
Marker
0 hr
1 hr
2 hr
3 hr
4 hr
24 hr
-ve

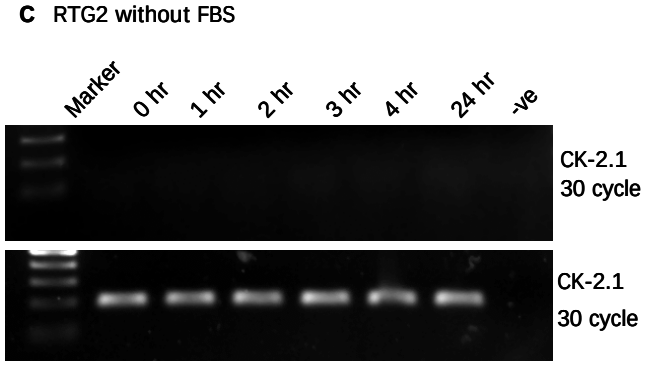

C RTG2 without FBS
Marker
0 hr
1 hr
2 hr
3 hr
4 hr
24 hr
-ve
CK-2.1
30 cycle
CK-2.1
30 cycle

Figure 3.10: RT-PCR with RNA samples collected from PHA treated RTS34ST cell line. (A) The genomic DNA was extracted and PCR was performed to check the allelic distribution of CK2 and CK2.1. The PCR products were run on a 1.5% agarose gel, stained with EtBr and visualized under UV-light. Five hundred ng (0.5μg) of total RNA was used to perform RT-PCR. (B) The RT-PCR results showing the transcript expression pattern. The top panel shows the results for the gene of interest whereas the bottom panel represents data the internal control EF1-α. The experiment was done with conditioned media enriched with 15% FBS.

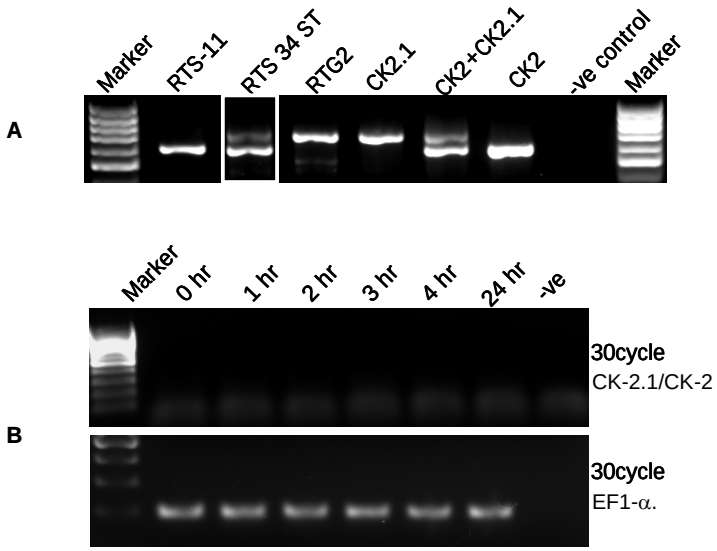

A
Marker
RTS-11
RTS 34 ST
RTG2
CK2.1
CK2+CK2.1
CK2
-ve control
Marker
B
Marker
0 hr
1 hr
2 hr
3 hr
4 hr
24 hr
-ve
30cycle
CK-2.1/CK-2
30cycle
EF1-α.

Figure 3.11: Western blot analysis of the protein extract collected from PHA stimulated RTS-11. RTS-11 cells were grown in 6-well plates with and without FBS. Cells were collected after stimulation with PHA. After protein extraction, around 100μg protein was loaded onto a 15% SDS-PAGE gel, transferred to a nitrocellulose membrane. The membrane was probed with purified primary anti-CK-2 followed by secondary anti-rabbit HRP conjugated antibody. The detection was performed by the ECL plus western blotting reagents, which was followed by exposing for 10min at chemiluminescence setting of the Flurochem 8000. The top panel shows the Ponceau S staining of the membrane, whereas the bottom panel represents the Western blot. (A) RTS-11 grown in the presence of FBS. A 30kDa faint band is visible at 4hr, 24hr, and 48 hr after stimulation. (B) RTS-11 cells were grown in the absence of FBS. Again, a 30kDa band is present in all the samples. Although after 4hr the band becomes less strong.

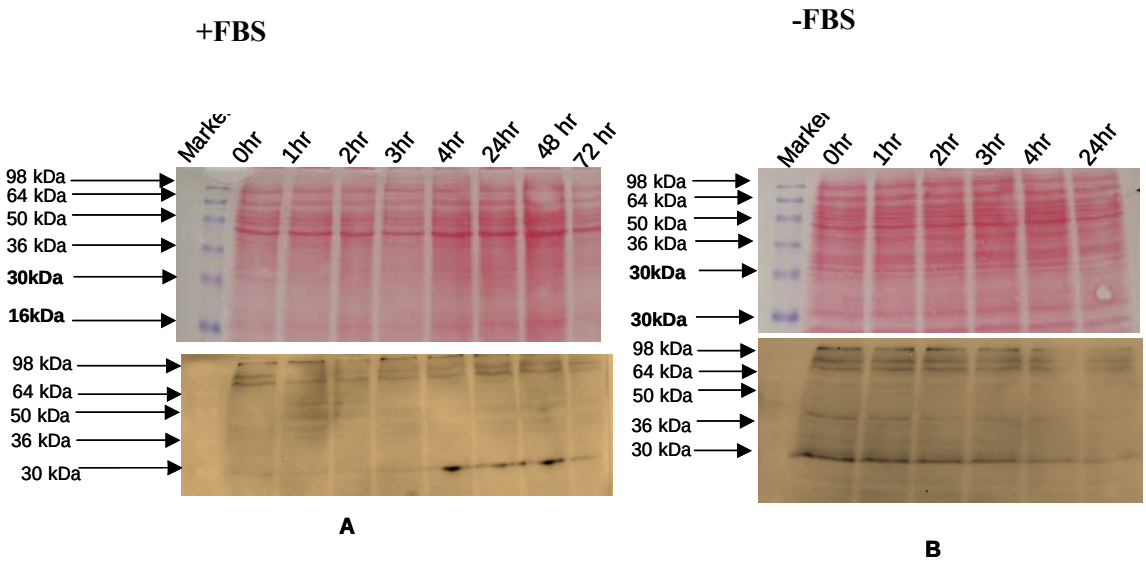
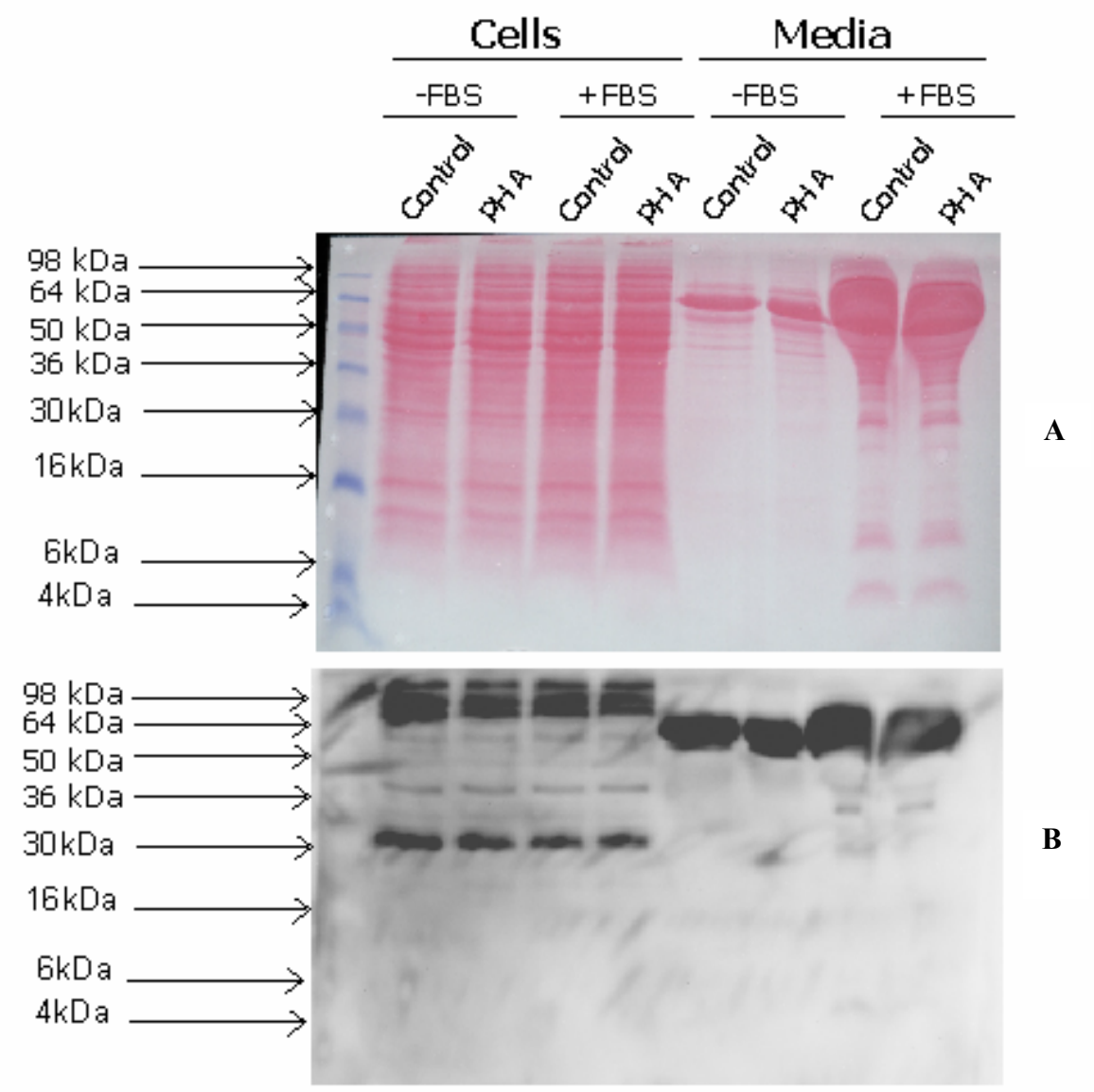

+FBS
-FBS
Marker
0hr
1hr
2hr
3hr
4hr
24hr
48 hr
72 hr
98 kDa
64 kDa
50 kDa
36 kDa
30kDa
16kDa
30 kDa
A
B

Figure 3.12: Western blot analysis of the protein samples collected from RTS-11 cells and the media. $1.5x10^7$ cells/well were cultured in 1ml media in a 12well culture plate. Again one well served as a control and another as an experimental. The experimental well was treated with PHA. The RTS-11 cells were grown with and without FBS. The cells were harvested after 24hrs, protein was extracted and the samples were run on a 15% polyacrylamide gel and transferred onto a nitrocellulose membrane. The primary antibody was added at a final dilution of 1:50; secondary antibody was added at a 1:1000 dilution; and the detection was carried out using ECL plus western blot detection system. (A) The Ponceau S staining of the membrane to show the equal loading of the sample. (B) The Western blot, which was probed with purified anti-CK-2 antibody to a 1:50 dilution.


Cells
Media
-FBS
+FBS
-FBS
+FBS
Control
PHA
Control
PHA
Control
PHA
Control
PHA
98 kDa
64 kDa
50 kDa
36 kDa
30kDa
16kDa
6kDa
4kDa
A
98 kDa
64 kDa
50 kDa
36 kDa
30kDa
16kDa
6kDa
4kDa
B

Upon harvesting the cells, protein was extracted. About 100ug protein was loaded to a 15% SDS-PAGE gel in order to perform Western blot. Samples from both cell extract and media were used for Western blot. It appears from the blot (figure 3.11) that, a very low level of CK-2 (30kda band) is produced at 4hr post stimulation, which seems to disappear after 72 hrs in RTS-11 cells cultured in the media with FBS. However, the proteins expression in the cells that were cultured in the media lacking FBS seems to show a different trend. A very low level of CK-2 (a 30kda band) is expressed, which tends to disappear after 4hrs. This is interesting since we have seen the similar pattern in the transcript expression of RTS-11 cells (-FBS). However, the pattern for the protein expression in cells grown in the presence of FBS does not coincide with the transcript expression. Although the transcript level is the highest in the control cells it drops down after treatment, but protein is expressed after 4hrs; however, the expression is very low. Nevertheless, in order to reach to any conclusion about the expression pattern, the experiment needs to be repeated a few more times The media samples were also used to carry out Western blot, but no expression was detected although being a secreted protein CK-2 is predicted to be found in the media separated from the cells.

Looking at the initial data, a separate experiment was conducted where about $1.5x10^7$ cells were cultured in 1ml of L-15 media in order to detect CK-2 in the media. The cells were treated with PHA for 24hr and both cells and media samples were collected. One well also served as a control group. In figure 3.12, the first two lanes represent protein expression in RTS-11cells cultured without FBS. It appears that the expression of protein was little bit higher in control sample, which does match with the transcript result where we see a higher level of expression in control sample. The RTS-11 cells cultured in FBS show a similar expression of CK-2 protein, which is different from what we have seen before (figure 3.12). Along with the presence of 30kDa band, a few bands with larger molecular mass are visible in all the cell samples suggesting the fact that the CK-2 can have different level of glycosylation. A 64kDa distinct band is present in both media samples collected from experiment with or without FBS. An extensive discussion on this topic is presented later.

Repetition of the experiment could explain little bit more about the trend. Also, purification of the bands followed by 2D gel electrophoresis is another possible alternative to know more about the bands visible in the blot. However, the unstable nature of chemokine makes it very difficult to study since it degrades very fast, which is also a problem for storage of protein and using for repeat experiments.

3.6 Transcript Expression of CK-2 and CK-2.1 *In Vivo*

In order to detect *in vivo* expression of CK-2, several rainbow trout were injected with PHA. A total of 8 fish were given intravenous injection. All the fish were genotyped beforehand to determine the allelic expression. Fish with both CK-2 and CK-2.1 were used for this experiment. Six fish were given an intravenous injection at a final concentration of 50μg/250g fish PHA. Two fish were sacrificed at each time point: 4hr, 8h, and 24hr after stimulation. Fish tissue including, HK, Liver, PBL, Brain, Spleen, Intestine, Muscle, and Heart were taken. RT-PCR was performed on the total RNA from all the tissue samples. Unfortunately, most of tissues were used for the purpose of Western blot analysis; therefore, one group of fish was used for the analysis of transcript. Also, some tissue showed degradation of RNA after few months of storage at -80^0C.

Figure 3.13 shows the expression pattern of both alleles in brain and HK. It is interesting to see that the level of CK-2.1 is slightly higher in brain than CK-2. However, following treatment the expression of both CK-2 and CK-2.1 decreases. Similar trend can be noticed in HK; nevertheless, HK has slightly higher level of CK-2 than CK-2.1. Again, the expression of both CK-2 and CK-2.1 seems to be decreasing post injection.

The expression pattern of transcript in spleen is quite interesting, where we see a very low level of CK-2.1 compared to CK-2 (group 1). Four hour after the injection, the level of CK-2 increases but after 8hr, the level decreases. Fortunately, the RNA from another group of fish did not degrade; therefore, the RT-PCR was performed on the RNA from that group. The second group showed another interesting feature where they have no expression of CK-2.1 (group 2). Moreover, the expression of CK-2 increased after 4hr post injection and disappeared after that. This second trend is somewhat similar to the first one except the fact that first group had very little expression of both CK-2 and CK-2.1 after 8hr and 24hr post injection (figure 3.14).

The transcript expression in liver shows another interesting pattern. Expression of both CK-2 and CK-2.1 was only detected 24hr post injection. However, the level was too low (figure 3.14). Unfortunately, the RNA from PBLs degraded; therefore, the transcript expression was not detected in this tissue. Also, since one group of fish was used for most of the tissue, no statistical analysis was carried out.

Figure 3.13: RT-PCR using the RNA sample collected from PHA stimulated rainbow trout brain and HK tissue. Total RNA was collected from the tissue samples and used to perform RT-PCR. About 1ug RNA was used for the cDNA synthesis. PCR primers were specific to both CK-2 and CK-2.1 giving rise to two products with about 100bp difference. Also, primer specific to EF1-α was used to detect the expression pattern for a housekeeping gene. In order to provide better comparison, the cycle number was kept consistent for both the gene of interest and internal control (30cycles). (A) Transcript expression pattern in rainbow trout brain. (B) Transcript expression pattern in rainbow trout HK. The bar graph represents the ratios (Gene of interest/internal control) of the band intensity using NIH imager. Raw data can be seen in Appendix B.

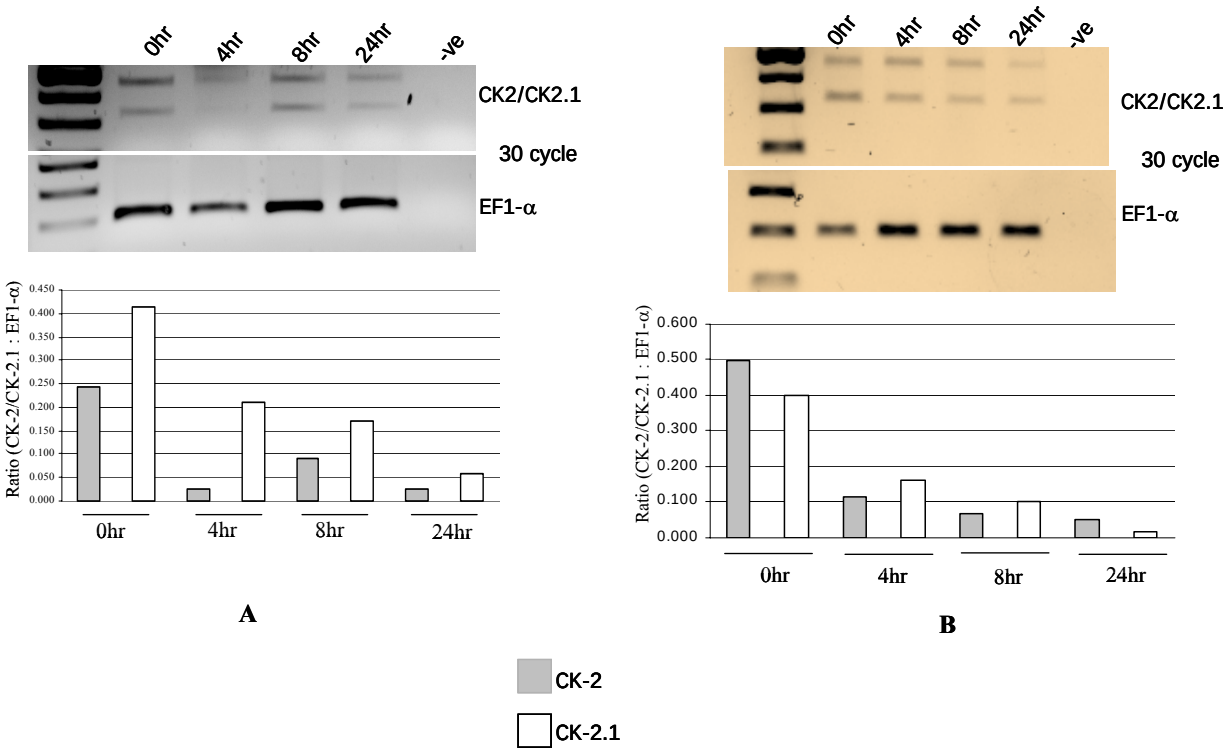
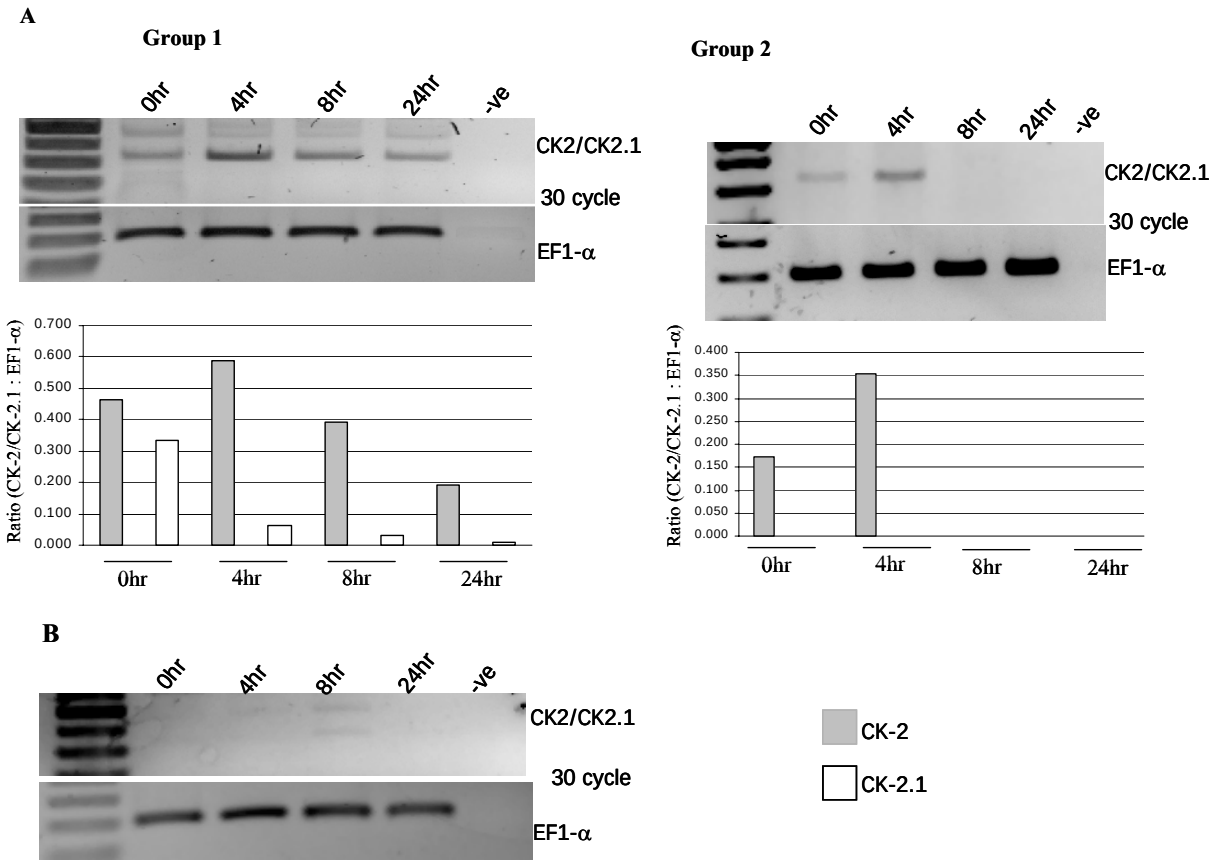

0hr
4hr
8hr
24hr
-ve
CK2/CK2.1
30 cycle
EF1-α
Ratio (CK-2/CK-2.1 : EF1-α)
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0hr
4hr
8hr
24hr
A
0.600
0.500
0.400
0.300
0.200
0.100
0.000
B
CK-2
CK-2.1

Figure 3.14: RT-PCR using the RNA sample collected from PHA stimulated rainbow trout spleen and liver tissue. Total RNA was collected from the tissue samples and used to perform RT-PCR. About 1ug RNA was used for the cDNA synthesis. PCR primers were specific to both CK-2 and CK-2.1 giving rise to two products with about 100bp difference. Also, primer specific to EF1-α was used to detect the expression pattern for a housekeeping gene. In order to provide better comparison, the cycle number was kept consistent for both the gene of interest and internal control (30cycles). (A) Transcript expression pattern in rainbow trout spleen. Two sets of data derived from the two groups of fish. (B) Transcript expression in pattern in rainbow trout liver. The bar graph represents the ratios (Gene of interest/internal control) of the band intensity using NIH imager. Raw data can be seen in Appendix B.


A
Group 1
0hr
4hr
8hr
24hr
-ve
CK2/CK2.1
30 cycle
EF1-α
Ratio (CK-2/CK-2.1 : EF1-α)
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0hr
4hr
8hr
24hr
Group 2
0hr
4hr
8hr
24hr
-ve
CK2/CK2.1
30 cycle
EF1-α
Ratio (CK-2/CK-2.1 : EF1-α)
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0hr
4hr
8hr
24hr
B
0hr
4hr
8hr
24hr
-ve
CK2/CK2.1
30 cycle
EF1-α
CK-2
CK-2.1

3.7 Expression of CK-2 *in vivo* at the Protein Level

Initially two fish were given intravenous injection at a final concentration of 50μg/250g fish PHA. The treated fish were sacrificed after 24hrs and HK, Liver, PBL, Brain, Spleen, Intestine, Muscle, and Heart were collected. A Western blot analysis (figure 3.15) represents signals in only few tissues. However, most of the bands were larger in size than expected. Among all the tissues a 36kDa band is visible in liver, a 98kDa band is present in brain, whereas a 250kDa band is present in HK. The bands represent proteins larger than the expected size (≤30kDa for CK-2 and ≤35kDa for CK-2.1). The presence of glycosylated mucin stalk could give the extra weight to the proteins causing them to be bigger than actual size. However, a more explicit explanation of the protein bands is provided in the discussion.

However, the time course experiment (as described in section 3.6) with rainbow trout was unable to show presence of any protein in most of the tissues. Only brain showed a consistent signal all the time. However, an interesting trend was noticed with this tissue. Brain showed a 98kDa band every time point post injection. A similar blot was probed with anti-CK-2 that was purified via sulfolink column containing mucin protein. Interestingly, when the blot was probed with antibody only to chemokine domain, a 30kDa band was noticed (figure 3.16). Similar results were seen from the tissue samples from another group of fish (Appendix C. figure C3). More details on this can be found in the discussion.

3.8 Chemotaxis Assay

3.8.1 Migration of PBLs toward CK-2, β_2M, ZAS, and PBS

In order to examine the function of CK-2, a chemotaxis assay was performed. PBLs were isolated from blood sample collected from rainbow trout. The lower chamber of the 48-well micro chemotaxis assembly was filled with protein and the top chamber was filled with PBLs suspended in L-15. After incubating an hour the membrane was washed and stained. The migrated cells were counted using a light microscope. β_2M was used as a negative control since it is a component of Major Histocompatibility Class I and theoretically it should not have any chemoattractant ability. It was produced using the same prokaryotic expression system. ZAS was used as a positive control. As seen in figure 3.17, there is significant migration of PBLs toward CK-2. Among three different concentrations, the peak was seen at 500ng/ml (17nm). Again, the migration of PBLs toward β_2Mwas

significantly lower than CK-2. It is quite visible from the graph that migration of PBLs is higher toward CK-2 than β_2M, which demonstrates that PBLs are not responding toward any other contaminating bacterial proteins. Also, there is a significant difference between the positive control ZAS and the negative control PBS. ZAS attracted more cells than PBS, which served as a negative control. PBS was used to count the number of cells migrate randomly toward any protein. The chemotactic index was also calculated; CK-2 with 500ng/ml concentration had a chemotactic factor of 8 whereas ZAS showed a chemotactic factor of 9 (Appendix B). A one-way ANOVA with a Fischer's LSD post hoc test was performed to determine the significance of the data. The data is significant with respect to the proteins (CK-2 and β_2M) ($p=0.007$); however, the data was not significant with respect to the protein concentration ($p=0.498$) although it appears from the figure that the migration is optimum at 500ng/ml (17nm) concentration. Figure 3.17 also demonstrates the light microscope images of the filter containing the migrated cells. The filter images for the membrane containing cells migrated toward ZAS and PBS are included to show the difference between positive and negative controls. The ZAS attracted many more cells than PBS, which served as a negative control to provide the random migration of cells though the filter.

3.8.2 Migration of RTS-11 toward CK-2, β_2M, ZAS, and PBS

The RTS-11 cells were also used to perform a chemotaxis assay. The RTS-11 cells were used right after they were split. As shown in figure 3.18, CK-2 is also able to attract RTS-11 cells. However, the peak of migration occurs at a concentration of 1μg/ml. This is quite interesting since previously it was shown that RTS-11 cells do produce CK-2. However, due to problems in repeating the assay using same condition as before, RTS-11 cells were plated overnight to use for chemotaxis. Interestingly, this second condition showed a peak in migration at 500ng/ml. The cells were too clumpy to count for the migration toward CK-2 at a final concentration of 10μg/ml. This also coincides with the data from the chemotaxis assay using the PBLs.

3.8.3 Blocking Experiment

In order to check the reliability of the chemotaxis assay, some blocking experiments were carried out. First, the PBLs were blocked with pertussis toxin. Pertussis toxin blocks the G-protein coupled receptors and inhibits the cell's ability to get attracted by chemokines. As seen in figure 3.19, due to the blockage, the PBLs are unable to migrate toward CK-2. Again, the PBLs were blocked with CK-

2, which blocked any receptor on the PBLs and inhibited the migration of the cells toward rCK-2. Lastly, rCK-2 was blocked with anti-CK-2 antibody, which diminished the chemotactic ability of CK-2 by blocking the epitopes. As expected, the cells are unable to migrate toward the rCK-2 since rCK-2 was blocked with anti-CK-2 antibody. In order to analyze the statistical significance a one-way ANOVA was conducted. The data was significant for both blocking experiments. There was significant decrease in migration when the cells were blocked with PTX. These blocked cells showed significant difference with non-blocked cells ($p=0.006$). Furthermore, the cells that were blocked with CK-2 showed significant decrease in migration when compared with the non-blocked cells ($p=0.001$); likewise, the data represented a significant value ($p=0.004$) for blocked CK-2with anti-CK-2 antibody. In short, the blocking experiments were successful in showing that the migration of cells were due to CK-2 and chemotaxis occurred when CK-2 bound to the 7 transmembrane, G-coupled receptor just like all other known chemokines.

Figure 3.15: Western blot analysis using the tissues from control and PHA stimulated rainbow trout. The tissue samples were used to extract protein, followed by running on a 15% polyacrylamide gel and transferred onto a nitrocellulose membrane. The primary antibody was added at a 1:50 dilution; secondary antibody at 1:1000; and the detection was carried out using ECL plus Western blot detection system. (A) The Ponceau S staining of the western blot to show the loading of the different protein samples. (B) The membrane was probed with anti-CK-2 antibody to a final dilution of 1:50. (C) A similar blot was probed with anti-CK-2 antibody blocked with rCK-2. (D) Ponceau S staining of the Western blot performed with protein sample form the tissues collected for control fish (non-stimulated). (E) This Western blot shows no signal for the presence of CK-2.

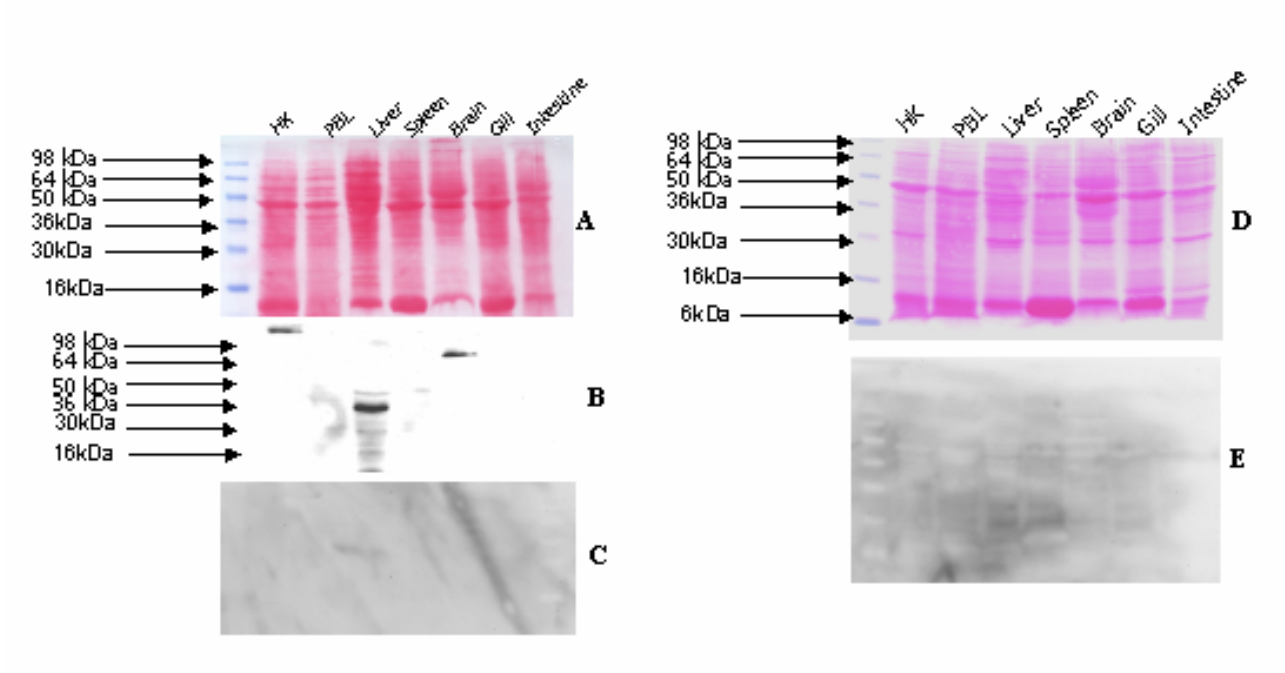
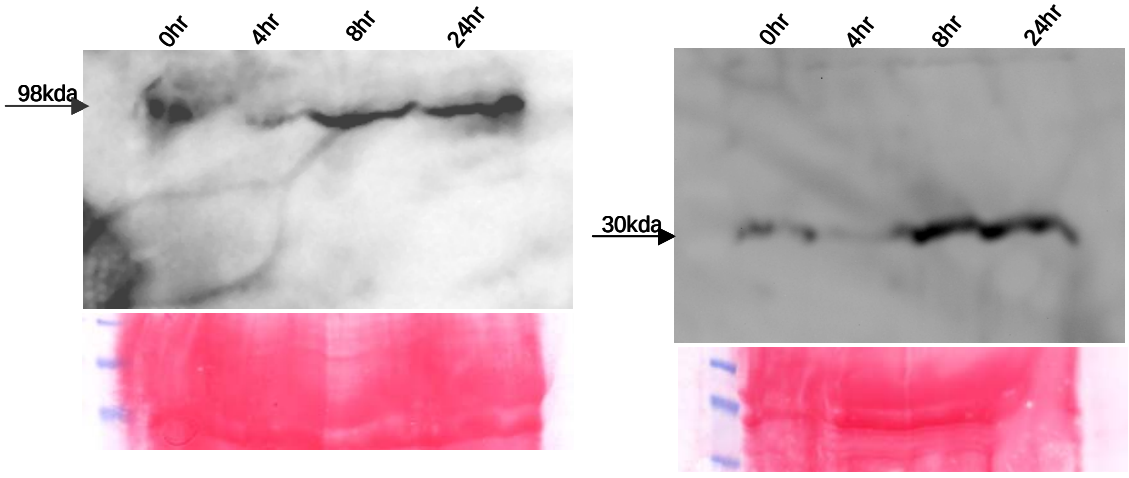

HK
PBL
Liver
Spleen
Brain
Gill
Intestine
98 kDa
64 kDa
50 kDa
36kDa
30kDa
16kDa
A
98 kDa
64 kDa
50 kDa
36 kDa
30kDa
16kDa
B
C
HK
PBL
Liver
Spleen
Brain
Gill
Intestine
98 kDa
64 kDa
50 kDa
36kDa
30kDa
16kDa
6kDa
D
E

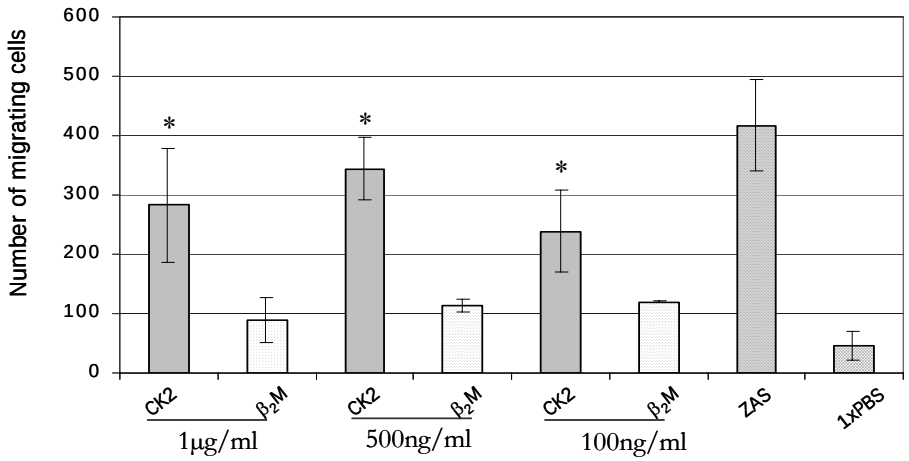
Figure 3.16: Western blot analysis of brain tissue extracts. A time course experiment was carried out by injecting the rainbow trout with PHA and sacrificing them after 4hr, 8hr, and 24hr. Protein extract from the brain tissue was used to perform Western blot analysis. The samples were separated on a 15% acrylamide gel. The top panel shows the Ponceau S staining of the nitrocellulose membrane; whereas the bottom panel shows the Western blots. The primary antibody was added at a final dilution of 1:50; secondary antibody at 1:1000; and the detection was carried out using ECL plus Western blot detection system. The top panel shows the Western blot, whereas the bottom panel shows the Ponceau S staining of the same membrane. (A) The membrane was probed with purified anti-CK-2 antibody at a final dilution of 1:50. (B) A second blot that was probed with antibody specific toward chemokine domain.



A **B**

Figure 3.17: Chemotaxis assay using PBLs. PBLs were isolated from rainbow trout blood sample and diluted to a final concentration of $1x10^7$cells/ml in L-15 medium. All the proteins were diluted to three different concentrations, including, 1μg/ml, 500ng/ml, and 100ng/ml. ZAS served as a positive control, while PBS served as a negative control. The proteins were loaded onto the lower chamber, whereas the cells were placed into the upper chamber. The whole assembly was incubated for 1hr at RT. The number of cells migrated was counted using a light microscope; total five fields were counted. The data was used to plot a bar graph. (A) The bar graph shows the average of three individual experiments. The error bars represent +/- standard error of the average. The data was transformed by taking the natural logarithm (ln) in order to compensate for the variations due to the use of different fish every time and other factors that might not have been consistent throughout the experiment (temperature, the handling of cells, etc.). Statistical analysis was conducted using a one-way ANOVA with a Fischer's LSD post hoc test. For PBLs, the data was significant with respect to the proteins (CK-2 and β_2M) ($p=0.007$); however, the data is not significant with respect to the protein concentration ($p=0.498$). (B) Light microscope images showing the field view for the positive (ZAS) and negative control (PBS). The magnification is 400x. (*) Represents the significant change.

A


Number of migrating cells
600
500
400
300
200
100
0
*
*
*
CK2
β2M
CK2
β2M
CK2
β2M
ZAS
1xPBS
1μg/ml
500ng/ml
100ng/ml

B

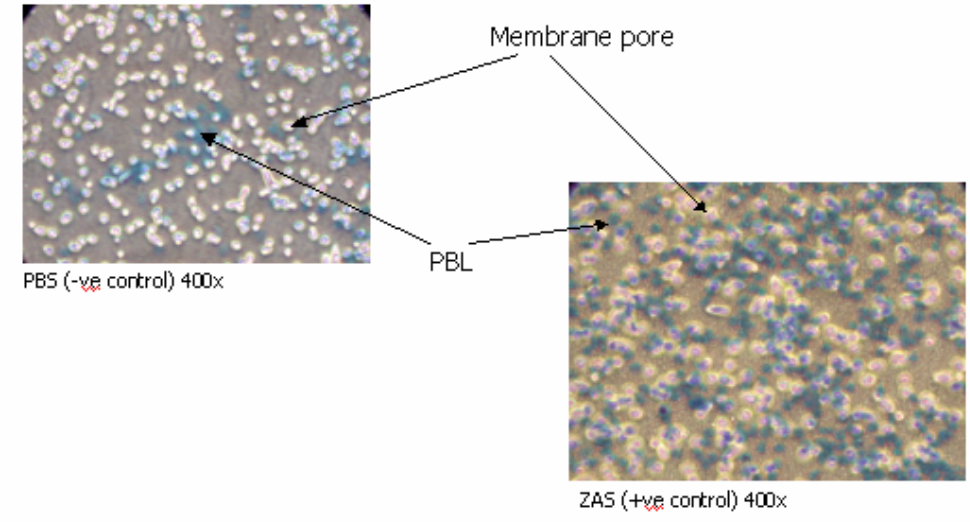
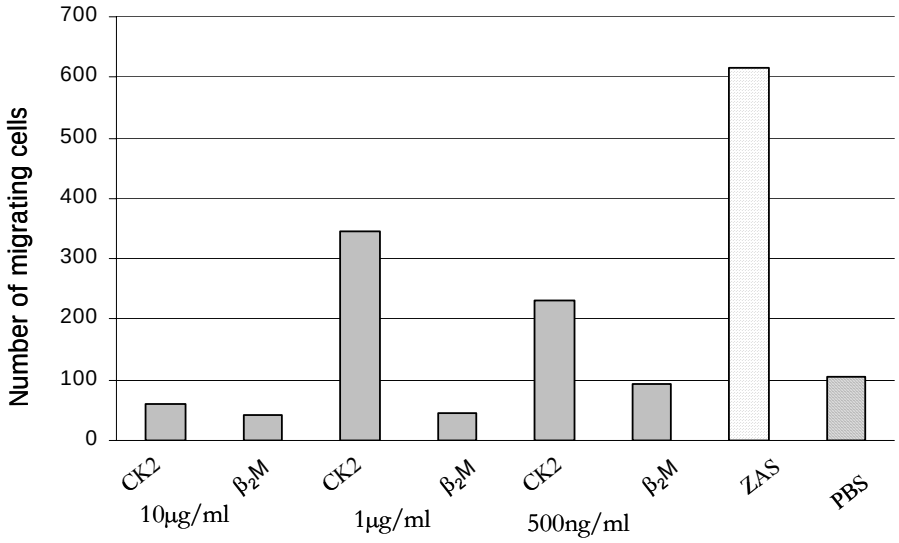
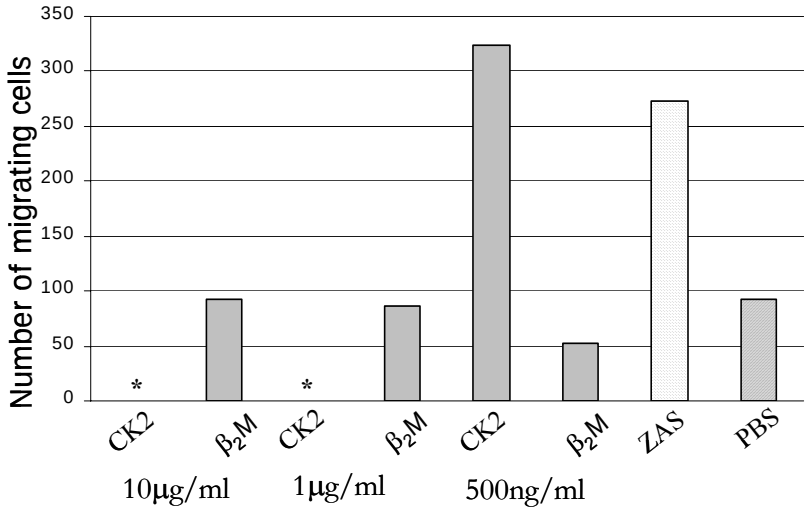

Membrane pore
PBL
PBS (-ve control) 400x
ZAS (+ve control) 400x

Figure 3.18: Chemotaxis assay using RTS-11. RTS-11 cells were diluted to a final $1x10^7$cells/ml in L-15 media. All the proteins were diluted to three different concentrations, including, 10μg/ml, 1μg/ml, and 500ng/ml. ZAS served as a positive control while PBS served as a negative control. The proteins were loaded onto the lower chamber whereas the cells were placed into the upper chamber. The whole was incubated for 1hr at RT. The number of migrating cells was counted using a light microscope; total five fields were counted. The data was used to plot a bar graph. (A) Chemotaxis assay with RTS-11 right after they were split. (B) Chemotaxis assay with RTS-11 after they were split and plated overnight and used for chemotaxis.

A



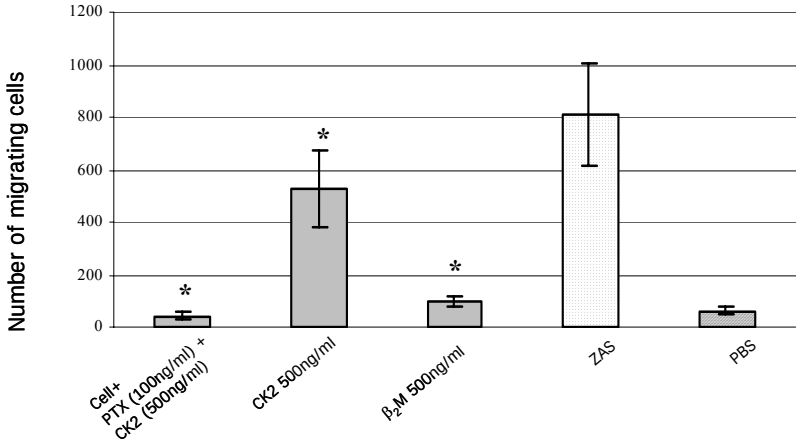
B



* Too clumpy to count

Figure 3.19: Blocking experiment. (A) The PBLs were mixed with PTX and incubated for 1hr. Following incubation, the cells were washed two times with PBS and resuspended in L-15 at a final concentration of $1x10^7$cells/ml. These cells were used for chemotaxis using CK-2 at a final concentration of 500ng/ml. All the controls were used to check the reliability of the experiment. After incubation, the number of migrated cells was counted. The bar graph represents the average of three individual experiments. (B) PBLs were blocked with CK-2 by incubating PBLs with CK-2 for 1hr. The cells were then washed; diluted to $1x10^7$cells/ml; and used for chemotaxis. The CK-2 was used at a final concentration of 500ng/ml. Also, the rCK-2 was blocked with anti-CK-2 antibody and used for chemotaxis using PBLs. Like other experiment, all the controls were used. The bar graph shows average of two separate experiments. The error bars represent +/- standard error of the average. One-way ANOVA was performed to detect the statistical significance. The cells blocked with PTX showed significant difference with non-blocked ($p=0.006$). Also, the data was significant for the cells blocked with CK-2 ($p=0.001$) as well as when CK-2 protein was blocked with anti-CK-2 antibody ($p=0.004$). (*) Represents the significant change.

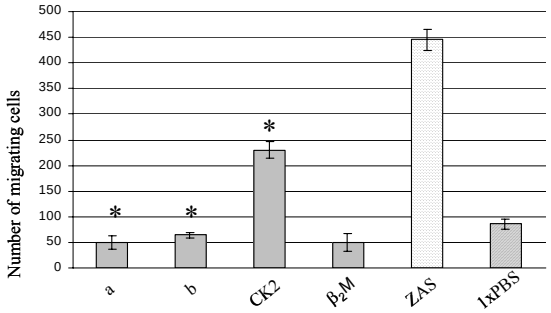
A



B

(a) Lower Chamber-CK-2
Upper chamber- Cells blocked with CK2

(b) Lower Chamber-CK-2 blocked with antibody
Upper chamber- Cells



Chapter 4
Discussion

Rainbow trout CK-2 is the main focus of this research. As discussed earlier this is a member of CC chemokine. However, not too much is known about this molecule other than the fact that this is the only CC chemokine with a mucin stalk in any organism. A very similar molecule is CK-2.1; however, the identity of this molecule was still in question. Therefore, one of the major objectives of the thesis was to identify whether CK-2.1 was an allele or a separate gene. Furthermore, the transcript expression of both CK-2 and CK-2.1 was also examined both *in vivo* and *in vitro*. Anti-CK-2 antibody was developed in order to detect the protein expression *in vivo* and *in vitro*. Lastly, a chemotaxis assay was performed to analyze the function of the chemokines. Here we present the data including both transcript and protein expression *in vivo* and *in vitro* along with a functional assay.

4.1.1 Allele vs. Gene

In order to determine whether CK-2.1 was an allele of CK-2 or a separate gene, genomic PCR and Southern blotting were performed. The genomic PCR on samples from several rainbow trout using primers flanking the extra mucin stalk region shows that CK-2.1 is an allele of CK-2 but not a separate gene. If CK-2.1 were a separate gene, all the fish that were genotyped would show the presence of both genes (CK-2 and CK-2.1) in their genome. Since individual rainbow trout showed different compositions of the two alleles, CK-2.1 is an allele of CK-2. The distribution of the two alleles in the fish typed is as follows: 20% homozygous for CK-2, 36% homozygous for CK-2.1, and 44% heterozygous (both CK-2 and CK-2.1). Theoretically, if two parents are heterozygous for any trait the ratio of the offspring should be 1:2:1, which represents 25% homozygous for dominant allele, 25% homozygous for recessive allele and 50% heterozygous. The observed results follow almost the similar trend because a random mating should produce results similar to a cross between two heterozygotes according to Hardy-Weinberg law. Moreover, the banding pattern in the Southern blot (figure 3.2) analysis shows variation due to the variability in the fish genome. Each individual fish has genomic structure slightly different than the others due to the presence of a little bit longer intron. Previously, it has been postulated that there could be two copies of CK-2 in rainbow trout, which supports the presence of two bands in some rainbow trout genomes (Liu *et. al.* 2002). However, the *Eco*RI digest of the genomic DNA shows one strong band in all three fish, which strongly suggests there could be only one copy of the gene. The presence of multiple gene copies in some fish after RE

digest could be due to the enormous variability in the genome of rainbow trout. In the end, the data presented here lead to the conclusion that CK-2.1 is an allele of CK-2. Also, depending on the individual, there could be one or two copies of the gene.

4.1.2 Tissue Expression Pattern of CK-2 and CK-2.1 at the Transcript Level

In order to examine the tissue expression pattern of the CK-2 and CK-2.1 transcript, both *in vivo* and *in vitro* experiments were carried out. The *in vivo* experiment involved injecting healthy rainbow trout with PHA intravenously (IV). Several fish tissues including brain, HK, PBL, liver and spleen were analyzed. Only rainbow trout containing both the CK-2 and CK-2.1 alleles were used for these experiments in order to see if stimuli had different effects on each allele. Although two fish were sacrificed at each time point, tissues from only one fish per time point was analyzed due to the degradation of RNA in some samples. Again, no RNA was detected in PBLs. However, analysis on spleen was carried out using tissues from both fish at every time point.

Beginning with brain tissue, the transcript level of both CK-2 and CK-2.1 shows a decreasing pattern over time following stimulation. However, brain contains a slight higher level of CK-2.1 than CK-2 (figure 3.13). The HK tissue expression pattern showed a decreasing trend similar to brain tissue. However, the level of CK-2 and CK-2.1 varies slightly. At 0hr, the CK-2 level is slightly higher than CK-2.1. At 4hr and 8hr post stimulation the transcript level decreases; however, the level of CK-2.1 is slightly higher than CK-2. Lastly, 24hr post stimulation shows the lowest amount of both CK-2 and CK-2.1, but this time CK-2 level is slightly higher than CK-2.1. It seems like two alleles are behaving differently upon stimulation.

Interestingly, spleen tissue (figure 3.14) represents little different expression pattern of CK-2/CK-2.1. An increasing pattern of CK-2 is noticeable in spleen. One group of fish showed no expression of CK-2.1, while the other group had lower level of CK-2.1 than CK-2. At 4hr post stimulation the level of CK-2 seemed to increase in both groups. In the group, that did not express any CK-2.1, the transcript for CK-2 disappeared after 8hr. The second group of fish showed a similar trend in which the CK-2 transcript level decreased after 8hr but with very low CK-2.1 expression. However, the decreasing trend of CK-2.1 was much faster than CK-2. The different expression pattern could be due to inter-individual variation. Lastly, liver tissue also showed as interesting expression pattern with a very low level of CK-2 and CK-2.1 expression at 8hr post-injection.

It is not quite clear, why brain possessed higher levels of CK-2.1 than CK-2, while in other tissues the level of CK-2 was a little higher than CK-2.1. On the other hand, spleen showed a very low level of CK-2.1, and expresses a lot more CK-2. Also, the expression pattern of CK-2/CK-2.1 in HK is little bit different from other tissues. It seems that CK-2 and CK-2.1 are preferred by specific tissues, which suggests that the two alleles have slightly different functions. However, any of these assumptions need to be further examined by performing the same experiment with more fish both homozygous and heterozygous. Looking at these data, it can be concluded that the expression of CK-2 and CK-2.1 varies depending on the tissue.

It is still not clear how exactly PHA provides an immune response by activating lymphocytes. However, various hypothesis have been proposed, among those the most significant one states the fact that PHA can work as an antigen. PHA binds to the specific receptors on the lymphocytes; nevertheless, the receptors are different from antigen receptors (Mackler *et. al.* 1972). In one study, Yamashiro *et. al.* (2000) showed that supernatant of PHA stimulated peripheral blood mononuclear cells (PHA-sup) contains tumor necrosis factor alpha (TNF-α). This PHA-sup has been shown to be responsible for the maximal monocyte chemoattractant protein 1 (MCP-1). MCP-1 is a human CC chemokine and attracts monocytes; MCP is secreted by monocytes, lymphocytes, endothelial cells, and smooth muscle cells. TNF-α is one of the major cytokines primarily responsible for inducing chemokines (Yamashiro *et. al.* 2000). TNF-α has been shown to be expressed in PHA –stimulated rainbow trout (Laing *et. al.* 2001). In addition, Zou *et. al.* (2005) showed an induced expression of interferon gamma (INF-γ) in rainbow trout head kidney leukocytes by stimulating them by PHA (Zou *et. al.* 2005). INF-γ is a cytokine and is mainly secreted and synthesized by Th1 cells and NK cells, and acts on macrophages, NK cells, and T cells. Several human studies have reported a synergistic effect between TNF-α and INF-γ on the transcription of various genes. A similar event might have been happening in the rainbow trout as well. However, this possibility has to be further addressed, which could be quite interesting since it could present a parallel event in fish.

As mentioned previously, fish lack discrete lymph nodes or a lymphatic system. Spleen and head kidney are the two major lymphoid organs in fish (Press *et.al.* 1994). That could possibly explain why there is an upregulation of CK-2 in spleen at 4hr post injection. Up-regulation of CK-2 is probably necessary to recruit leukocytes to the lymphoid organs. It has been shown that the secondary lymphoid-tissue chemokine, SLC, (a novel human CC chemokine) has selectivity for T lymphocytes. Its expression is high in lymphoid tissues and its biological activity (chemotaxis, Ca^{2+} influx)

increases after PHA stimulation (Williamann *et. al.*1998). Moreover, CCR7, which is the specific SLC receptor on T-lymphocytes, has shown to be upregulated by PHA stimulation (Williamann *et. al.*1998).

In addition, it is important to mention that transcript for CK-2 and CK-2.1 is very unstable because of the presence of RNA instability motif (AUUUA) in both the 5' and 3' untranslated regions. While the function of the presence of adenylate uridylate-rich (AU-rich) (ARE) motifs at 5' end is yet unknown, the presence of them at the 3' untranslated region accounts for the short half-life of chemokine mRNAs, as well as mRNAs from many other immune system genes (Liu *et. al.* 2002 and Bakheet *et. al* 2001). However, Xu *et. al.* (1997) has postulated that the interplay of the 5' AU-rich region and the 3' cluster of AUUUA motifs are required for the ultimate rapid and processive degradation of mRNAs. It is known that the AREs lead to the rapid turnover of mRNAs encoding early response genes that regulate cellular growth as well as immune response to exogenous agents such as microbes, inflammatory and environmental stimuli. These mRNAs have been functionally attributed to a restricted number of genes such as certain hematopoietic cell growth factors (e.g. granulocyte–monocyte colony stimulating factor, GM-CSF), interleukins (IL), INF, TNF-α and some proto-oncogenes (c-fos, k-ras and pim-1). Therefore, a general feature of these special ARE-mRNAs is that they are short-lived; thus, they rapidly disappear once their critical role in gene regulation ceases (Bakheet *et. al* 2001). That is the reason why most of the cytokine mRNAs possess multiple copies of AUUUA pentanucleotides that cluster together; for instance, the GM-CSF ARE, TNF-α ARE, and IL-3 ARE. Consequently, the decreasing trend of the CK2 and CK2.1 transcripts seen in this study could be due to the combination of decreased transcription combined with the short-half life, or simply a decrease in mRNA half-life.

A parallel study was performed *in vitro* using primary cultured HKLs and PBLs treated with PHA. The HKLs showed an upregulation of CK-2 and CK-2.1 after the stimulation (figure 3.7). This upregulation contrasts the findings of the *in vivo* study where there was a down-regulation of CK-2 and CK-2.1 24hr post injection. HKLs derived from heterozygous fish showed an interesting expression pattern because they expressed a lower level of CK-2.1 than CK-2. Neither CK-2 nor CK-2.1 was upregulated *in vivo*, despite being upregulated after direct stimulation by PHA *in vitro*, possibly due to the difference in the route of stimulation. The cells were treated directly by the addition of PHA, whereas *in vivo* study dealt with injecting the fish and assuming that the tissues would achieve the similar treatment. Also, the different expression could be due to fact that in the *in*

vivo experiment the RNA was made from cells immediately after removal from the fish whereas in the *in vitro* study the cells were cultured for 24hr, which may have led to the altered expression patterns. Moreover, the condition medium was enriched with antibiotics, which could also contribute to the differential expression pattern. In addition, it could also be possible that the cells producing CK-2 migrated out of the HK and went to some other tissues, whereas in the culture plate it was impossible. The primary cultured PBLs did not show that much upregulation. The *in vivo* transcript expression of PBLs was not detected due to the RNA degradation.

The second half of the *in vitro* study using the RTS-11 cell line was also interesting. Depending on the presence or absence of FBS in the culture media, the RTS-11 cells showed a slightly different CK-2/CK-2.1 expression pattern (figure 3.8). In the presence of FBS, the CK-2/CK-2.1 expression level decreased after the stimulation. However, in the absence of FBS there was an upregulation of CK-2 after 4hr followed by a decrease after 24hrs. This result matches the *in vivo* expression of CK-2 in spleen where the mRNA level increases 4hr post stimulation and then decreases after 8hr. This is quite interesting because RTS-11 is a macrophage like cell line which was derived from spleen tissue.

The RTG-2 and RTS34ST cell lines were tested but do not express either of the two CK-2 alleles, although RTG-2 has a genomic copy of CK2.1 and RTS34ST contains both CK-2 and CK2.1 in its genome (figure 3.9 and 3.10). RTG-2 is a salmonid fish cell line derived from the gonad of rainbow trout (Wolf and Quimby 1962), whereas RTS34ST is a rainbow trout stromal cell line (Ganassin and Bols 1999). Therefore, it can be concluded that the gonads and stromal cells in rainbow trout probably do not express CK-2 or CK-2.1. Unfortunately, the same tissues were not analyzed *in vivo*, which could have provided more insights about the expression pattern.

4.1.3 Expression of the CK-2 and CK-2.1 Protein Product *In Vivo* and *In Vitro*

The same fish from the RNA study were used for the Western blot analysis. The newly produced anti-CK-2 antibody was shown to cross-react with CK-2.1 (figure 3.6); therefore, heterozygous fish were chosen so that the expression pattern of both CK-2 and CK-2.1 could be detected. However, due to the very low abundance of CK-2 and CK-2.1 protein in trout tissues, it was really difficult to detect the protein product by Western blot. However, after optimizing several factors, it was possible to detect some expression in a few tissues. The rCK-2 and rCK-2.1 were found to be about 30kDa and 35kDa, respectively when it contained the is-tag (3kDa). However, without his-tag, a band of less than 30kDa for CK-2 and 35kDa for CK-2.1 were expected. To begin, a 36kDa band was detected in

liver tissue, while a 98kDa band and a 250kDa band were also visible in brain and HK, respectively (figure 3.15). All the proteins found in these different tissues were larger than expected. Also, only one band was seen in all those tissue, which made it complicated to identify if the band was for CK-2 or CK-2.1. Nevertheless, it could be possible that only one protein is expressed and that could be either CK-2 or CK-2.1. Also, the difference between two proteins is not very large, therefore, it could be possible that CK-2 and CK-2.1 are both expressed but they are stacked onto each other when ran on the polyacrylamide gel.

One possible reason for higher molecular mass could be the presence of sugar residues on the mucin stalk, which are known to be heavily glycosylated by *O*-glycans in the proteins. There are about 43 possible *O*-glycosylation sites in the CK-2 mucin stalk and 53 in CK-2.1 mucin stalk. The presence of so many sugar residues might contribute to the large molecular mass of the protein found in different tissues. The different sizes could be due to the different degrees of glycosylation in different tissues. Interestingly, it has been well established that the terminal glycosylation sequences differs from tissue to tissue and between cell types within each tissue and these sequences are differentially expressed during early development (Van den Steen *et. al.* 1998; Paulson 1989). Also, synthesis of glycoprotein sugar chains represents post-translational modification, which includes the addition of single *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) monosaccharides to serine or threonine residues. Unlike traditional *N*-linked glycosylation of extracellular proteins, *O*-GlcNAcylation is dynamic, reversible, and responsive to extracellular stimuli (Zachara and Hart 2004). The ultimate structure of *O*-linked glycosylated proteins is dependent on the glycosyltranferase since they produce the glycoproteins. Thus, variation in sugar chain composition between cells and tissues is believed to be due to the differential expression of the many glycosyltranferases (Rademacher 1988). This strongly suggests why different tissue sample showed different sizes proteins corresponding to different level of glycosylation.

Most of the tissues from the rainbow trout in time course experiment did not show and protein (CK-2/CK-2.1) expression. The only tissue, which showed presence of either CK-2 or CK-2.1 protein, is brain. Brain showed a consistent 98kDa band after the stimulus. In order to analyze further, the same protein extract was used for a Western blot probed with the antibody specific for only the chemokine domain. This time the same tissue samples showed a protein band with a molecular mass of 30kDa. It may be that the two different antibody preparations are recognizing the two different glycosylation stages of CK-2 or CK-2.1. There is a possibility that, antibody toward chemokine

domain and mucin stalk is recognizing the glycosylated version of the protein and when the antibody toward mucin stalk is removed by purification, the residual chemokine domain antibody recognizes a non-glycosylated form of the protein (figure 3.16).

CK-2 has about 25% identity with the human CX_3C chemokine fractalkine (or neurotactin, a murine mucin stalk-containing a mucin stalk) and Pan *et. al.* has shown that neurotactin messenger RNA is predominantly expressed in normal murine brain. Also, fractalkine protein expression has been shown to be upregulated in activated brain microglia of mice with experimental autoimmune encephalomyelitis (EAE), as well as in mice treated with lipopolysaccharide (Pan *et. al.* 1997). On the other hand, Schwaeblea *et. al* (1998) postulated that neuronal cells are the major source of fractalkine biosynthesis in the central nervous system (CNS). These researchers used rat and mouse models of EAE, and showed that there is a high abundance of fractalkine mRNA in neuronal cells and the plasticity of expression is not affected by EAE brain inflammation. Moreover, immunohistochemical analysis of human and monkey CNS tissues showed a prominent neuronal staining for fractalkine (Schwaeblea *et. al.* 1998). Looking at these data, it could be suggested that CK-2 might have the similar function as human fractalkine or neurotactin since it is abundant in rainbow trout brain.

An effort was made to identify whether the protein detected in brain tissue was CK2 or not using 2-dimensional (2D) gel and mass spectroscopy (MS). The 2D gel was supposed to separate a 98kDa band. This would be simple if it was a chemokine as they have a very high isoelectric point (pI=9.3 for CK2 and pI=9.49 for CK-2.1). However, a crude sample was used for this purpose, which made it harder to identify the protein on the gel as well as to run MS on the sample. To approach this problem, the brain extract was purified via affinity chromatography using sulfolink column containing anti-CK-2 antibody. However, the purified protein was not enough to perform a 2D gel; therefore, more brain tissue has to be collected in order to extract protein. Later, the purified sample can be analyzed via 2D gel and MS to identify the protein.

Moreover, deglycosylation of the native protein using different tissue (HK, brain, liver) extracts was performed using a mixture of deglycosidases including endo-α-*N*-acetylgalactosaminidase, α2-3, 6, 8, 9-neuraminidase, β 1,4-galactosidase, and β-N-acetylglucosaminidase to remove the *O*-linked glycosylation. However, this effort was unsuccessful. The problem with *O*-glycosylation is that the enzymes available to remove the sugar residues are very limited. Also, the monosaccharides need to cleave sequentially, which makes it difficult to remove the sugar residues. Any modification of the core structure can block the action of *O*-Glycosidase; therefore, it is necessary to know the structure

of *O*-glycosylated mucin stalk. There are several enzymes for removal of specific sugar residues; however, fucose and mannose directly *O*-linked to proteins cannot presently be removed enzymatically (Spiro and Bhoyroo 1974; Stults and Cummings 1993; and Chiba *et. al.*1977). In short, the deglycosylation procedure should be optimized so that it works well with the glycosylation on CK-2; also a positive control should be included that can confirm the efficiency of deglycosylation.

It is interesting to note that protein products were detected in tissues (HK, Liver, Brain) after 24hr of stimulation; whereas, the mRNA level was down regulated after 24hrs. This suggests that the protein is more stable than mRNA. *O*-Glycosylation has different biological effects on the protein products. The glycosylation can provide the stability of the proteins by preventing denaturation (Van den Steen *et. al.* 1998). Therefore, it might be possible that the protein product of CK-2 or CK-2.1 is more stable than mRNA. It could also be possible that the rate of mRNA degradation is faster than the rate of protein degradation, providing presence of protein at 24hr when mRNA level decreased. It is worth mentioning that it was difficult to repeat any Western blot analysis. Although the Ponceau S staining used to show intact protein bands, there was always problem detecting CK-2 or CK-2.1. It is quite possible that the proteins had tendency to degrade faster after extracting from tissue samples.

The *in vitro* study with RTS-11 showed the presence of a 30kD protein detected by the CK-2 antisera by Western blot. However, it is quite difficult to predict the expression pattern. As seen in figure 3.11 in the presence of FBS, RTS-11 shows expression of CK-2 at 4hr after stimulation. In the absence of FBS, the protein expression shows a decreasing pattern with time. However, another experiment using a lower volume of media in the culture wells showed slightly different results because CK-2 protein (30kDa) expression was visible in the presence or absence of FBS. In addition, a few high molecular weight proteins were also present suggesting the fact that those could be different forms of glycosylated protein (figure 3.12). Interestingly, the media (separated from the cells) from both conditions also contain a cross-reactive band around 64kDa. The FBS contains serum albumin (67kDa) (Dodson *et. al.* 2001), which suggests that the signal could be due to the cross-reactivity with the serum albumin in the media supplemented with FBS. However, it is quite interesting to see the presence of similar band in the conditioned media without the FBS. CK-2 is a secreted protein; thus, the protein should be present in the media in which RTS-11 cells were cultured. However, the size is different from the ones detected in the sample collected from the cells. In addition it is quite possible that the 30kDa band is a non-glycosylated form found in endoplasmic reticulum (ER). Unlike *N*-linked glycosylation, *O*-glycosylation occurs in Golgi apparatus (Van den

Steen *et. al.* 1998). Therefore, these proteins have to be transported from ER to Golgi. Thus, it is quite possible that the different sizes of protein visible in figure 3.12 could be the proteins present in different compartments giving different sizes along with different stages of the glycosylation.

Most secretory proteins are initially synthesized as relatively long-lived, inactive precursors, known as proproteins. However, these proproteins require further proteolytic processing in order to generate the active or mature proteins. Usually, the proteolytic conversion of a proprotein to the corresponding mature protein occurs in secretory vesicles as they move away from the *trans*-Golgi. During this process, mature secretory vesicles are formed by fusion of several immature ones containing proprotein, which is cleaved during this maturation process (Steiner *et. al.* 1984). Therefore, it is quite possible that CK-2, being a secretory protein might have been processed during secretion leading to a smaller version of the glycosylated form.

In short, the protein data from both *in vivo* and *in vitro* experiments suggest that further investigation is required to fully understand the expression of this protein. It was not quite clear which of the two proteins (CK-2/CK-2.1) was detected in the tissue. There might be possible protein degradation which may be related to the problem in detecting it in the tissues. The potential glycosylation lead to a lot of uncertainty as to what size protein band CK-2/CK-2.1 would produce on an SDS-PAGE gel. Most of the data could not be replicated due to these problems. Another problem associated with the difficulty in protein detection is the low expression level of chemokines in general in the tissues, making them hard to detect. The mRNA level was so low in tissues that for RT-PCR about 4 times more RNA was used for the amplification of chemokines than the internal control. Moreover, chemokines are secreted during immune function and all mammalian cells studied showed that they attract leukocytes at a very low concentration (in the nanomolar range). Therefore, in order to detect this low abundant protein, the detection system probably has to be more sensitive than the one used here.

4.1.4 Chemotaxis Assay

The major function of chemokines is to attract the leukocytes to either sites of injury and inflammation, or to secondary immune organs. In order to examine the chemotactic ability of CK-2, a chemotaxis assay was performed as described in Falk *et.al.* 1980. The data indicates that CK-2 can attract PBLs in a concentration dependant manner with an optimal concentration of 500ng/ml (17nm) (See figure 3.16). Moreover, there was no significant response to rβ_2M, which was produced in the

same prokaryotic expression system. This result indicates that the migration of PBLs is not due to any contaminating bacterial components. Also, ZAS was used as a positive control. ZAS contains the complement-derived activator C5a, which has ability to attract the leukocytes like chemokines (Smith *et.al.*1984 and Antrum *et.al.*1986). In mammals, C5a has been shown to be chemotactic for number of cells, including neutrophils, monocytes, T and B-lymphocytes (Ottonello *et. al.* 1999) and dendritic cells (Yang *et. al.* 2000). Likewise, in teleosts, it been shown that ZAS activated serum has the ability to attract trout leukocytes (Hamdani *et. al.* 1998). As a CC chemokine, CK-2 should be able to attract monocytes, lymphocytes, basophils, and eosinophils, the usual components of PBLs. Bazan *et. al.* showed that fractalkine can attract monocytes and T-lymphocytes but not neutrophils. CXCL16 has been shown to be chemoattractant for T-lymphocytes. However, the specific types of cells that are attracted toward CK-2 still need to be determined.

The blocking experiment (figure 3.18) proved that the chemotaxis was specifically due to CK-2. Blocking the cells with PTX inhibited the migration of PBLs, which proves that the migration of PBLs involves the binding of CK-2 to a G-protein coupled receptor on the leukocyte surface. PTX is a protein virulence factor produced by *Bordetella pertussis*. It is a noncovalently linked heterohexameric protein and functionally subdivided into two subunits. The A subunit (A protomer) is composed of a single peptide, which possesses ADP-ribosyltransferase activity and this can modify GTP-binding regulatory proteins (G proteins). ADP-ribosyltransferase affects signal transduction by ribosylation of the α subunit of trimeric Gi proteins. As we know, chemokine receptors are also G-protein coupled receptors; thus, PTX can modify their function leading to the blockage of the receptor activity. On the other hand, the B subunit of PTX is composed of pentameric protein complex. This confers cell surface binding specificity on the toxin and delivers the A protomer into the cell (Su *et. al.* 2001 and Kaslow and Burns 1992). In addition, the blocking of rCK-2 with the anti-CK-2 antibody also inhibited the migration of PBLs. This provides further clarification that CK-2 is the chemotactic factor and not anything else in the PBS applied to the bottom of the chemotaxis chamber.

Another interesting finding is that RTS-11, a macrophage/monocyte rainbow trout cell line (Ganassin and Bols 1998), migrated towards CK-2 (figure 3.17). Previously, RTS-11 showed expression of CK-2, and it is intriguing that RTS-11 cells are able to produce CK-2 as well as be attracted toward CK-2. . Chemokines are often secreted by activated macrophages. However, they are also secreted by endothelial cells, but not by leukocytes, suggesting that it is of particular importance for leukocyte recruitment from the blood (Mackay 1997). The RTS-11 cell line is made up of two different kinds of cells. One type shares the characteristics as macrophages and other type shares

characteristics with monocytes. Therefore, it could be possible that monocyte like cells are attracted by CK-2 and the macrophage like cell line produce CK-2. However, this possibility has to be analyzed further before definitive conclusions can be reached. Moreover, it has been shown that lymphotactin (C chemokine) is produced by NK cells, but they can attract both NK cells and T lymphocytes (Hedrick *et. al.* 1997). Therefore, it might be possible that CK-2 is produced by macrophages and they can also attract them.

4.1.5 Future Work

The chemotactic nature of CK-2 has been established from the above study by showing that it can attract leukocytes. However, the chemotactic function of CK-2 could be further studied with different recombinant proteins made from only the chemokine domain and mucin stalk region. Both protein products have been expressed during the above research project. However, previously it has been shown that the chemokine domain of mammalian fractalkine is responsible for the chemotaxis (Bazan *et. al.* 1997). Thus, it would be quite interesting to know if the rainbow trout share the same characteristics since it has similarity with the human fractalkine. Also, further investigation should focus on using a cell sorter to identify the cells that are attracted by CK-2. The expression of both CK-2 transcript and protein was quite interesting, but needs more study, perhaps using more sensitive techniques. Also, fish homozygous for the CK-2.1 and CK-2 alleles could be studied to examine the expression pattern of both message and proteins independently. Last but not least, a yeast two-hybrid assay could be established to find the receptor specific for CK-2.

4.1.6 Concluding Remarks

Teleost fish represent one of the most primitive groups of vertebrates, but they possess both innate and acquired immunity homologous to those of the mammalian immune system (Magor and Vasta 1998). Hence, studying fish is quite helpful for comparative immunology in order to examine the evolutionary relationship among organisms. This study was able to determine the function of CK-2 and establish the fact that it is indeed a chemokine. Moreover, both transcript and protein expression showed that this gene is not constitutively expressed, but rather is regulated in a complicated manner. Overall, these preliminary data helped us understand fish chemokines in general and will be helpful in examining the molecule further. In addition, any further knowledge might be helpful in designing a fish vaccine that would be beneficial to the fish health in aquaculture.

Appendix A

Abbreviations

Ampicillin	**Amp**
Beta- 2- Microglobulin	**β_2M**
Chemokine 2	**CK-2**
Chemokine 2.1	**CK-2.1**
Chloramphenicol	**Chl**
Chloroform-isoamyl alcohol	**CIAA**
Complementary DNA	**cDNA**
Diethyl pyrocarbonate	**DEPC**
Enzyme Linked ImmunoSorbent Assay	**ELISA**
Ethyl 3-aminobenzoate methanosulfonate salt	**MS-222**
Fetal Bovine Serum	**FBS**
Head Kidney Leukocytes	**HKL**
Hour	**Hr**
Interferons	**INF**
Interleukins	**IL**
Keyhole Limpet Hemocyanin	**KLH**
Luria Bertani	**LB**
Mass Spectroscopy	**MS**
Minute	**Min**
Monocyte chemoattractant protein 1	**MCP-1**
Penicillin	**Pen**
Peripheral Blood Leukocytes	**PBL**
Pertussis Toxin	**PTX**
Phenol-chloroform-isoamyl alcohol	**PCI**
Phosphate Buffered Saline	**PBS**
Phytohemaglutinin	**PHA**
Recombinant CK2	**rCK-2**
Restriction Enzyme	**RE**

Ribo Neucleic Acid	**RNA**
Room Temperature	**RT**
Seconds	**Sec.**
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis	**SDS-PAGE**
Streptomycin	**Strep**
Tumor necrosis factor alpha	**TNF-α**
Two-dimensional	**2D**
Zymosan Activated Serum	**ZAS**

Appendix B

Tables

Table B1: Raw data of the densitometry analysis for the transcript expression of CK-2/CK-2.1 in RTS-11. The expression was detected by RT-PCR. The ratios were derived by dividing the band intensity of CK/CK-2.1 by the band intensity of EF1-α. There were three separate experiments and an average was taken to plot the graph.

(a) The experiment was done with RTS-11 grown in media lacking FBS

Time	Band Intensity of CK-2 in $(pixels)^2$	Band Intensity of EF1-α in $(pixels)^2$	Ratios CK-2: EF1-α	Mean Ratio	Standard Deviation	Standard Errors
0 hr	108	211	0.512			
	69	187	0.369	**0.575**	**0.458**	**0.264**
	397	470	0.845			
1 hr	163	222	0.734			
	36	66	0.545	**0.635**	**0.133**	**0.077**
	175	280	0.625			
2 hr	87	194	0.448			
	97	111	0.874	**0.883**	**0.301**	**0.174**
	504	380	1.326			
3 hr	83	183	0.454			
	46	60	0.767	**0.708**	**0.221**	**0.128**
	246	272	0.904			
4 hr	105	212	0.495			
	82	93	0.882	**0.951**	**0.273**	**0.158**
	500	339	1.475			
24 hr	105	206	0.510			
	8	52	0.154	**0.248**	**0.252**	**0.145**
	17	216	0.079			

(b) The experiment was done with RTS-11 grown in media enriched with FBS. Again, average was taken for three individual experiments.

Time	Band Intensity of CK-2 in $(pixels)^2$	Band Intensity of EF1-α in $(pixels)^2$	Ratios CK-2: EF1-α	Mean Ratio	Standard Deviation	Standard Errors
0 hr	55	74	0.743			
	534	268	1.993	**0.995**	**0.898**	**0.519**
	49	196	0.250			
1 hr	31	139	0.223			
	437	431	1.014	**0.613**	**0.447**	**0.258**
	91	177	0.514			
2 hr	22	86	0.256			
	387	357	1.084	**0.517**	**0.492**	**0.284**
	34	162	0.210			
3 hr	17	116	0.147			
	323	426	0.758	**0.461**	**0.306**	**0.177**
	77	161	0.478			
4 hr	10	105	0.095			
	303	446	0.679	**0.458**	**0.317**	**0.183**
	88	147	0.599			
24 hr	14	82	0.171			
	182	354	0.514	**0.417**	**0.215**	**0.124**
	103	182	0.566			

Table B2: Results of the chemotaxis assay. A total five different fields per well were counted using a light microscope at 400x magnification. In order to calculate the chemotactic index, the number of cells that migrated in response to chemokines was divided by the number of cells that spontaneously migrated (toward negative control, PBS).

(a) Chemotaxis assay showing the migration of PBLs toward several proteins including positive (ZAS) and negative (PBS, β2M) controls.

PBLs	**Trial 1**	**Trial 2**	**Trial 3**	**Average**	**Std. Dev.**	**Std. Error**	**Chemotactic Index**
CK2 (1ug/ml)	407	94	349	283.33	166.51	96.14	6
β_2M (1ug/ml)	96	22	152	90.00	65.21	37.65	2
CK2 (500ng/ml)	339	439	255	344.33	92.12	53.18	8
β_2M (500ng/ml)	95	135	110	113.33	20.21	11.67	3
CK2 (100ng/ml)	375	160	182	239.00	118.29	68.30	5
β_2M (100ng/ml)	121	119		120.00	1.41	0.82	3
ZAS in PBS (10%)	572	349	330	417.00	134.57	77.69	9
PBS alone	13	30	92	45.00	41.58	24.01	1

(b) Blocking experiment. Migration of PBLs toward blocked rCK2. Also, cells blocked with CK2 were unable to migrate toward rCK-2.

	Trial 1	**Trial 2**	**Average**	**Std. Dev.**	**Std. Error**
Cell+CK-2	62	36	49	18.4	13
CK2+antibody	70	58	64	8.5	6
CK2 (500ng/ml)	246	215	230.5	21.9	15.5
β_2M (500ng/ml)	67	32	49.5	24.7	17.5
ZAS	466	424	445	29.7	21
PBS	96	75	85.5	14.8	10.5

© Blocking experiment. PBLs blocked with Pertussis Toxin showed very little migration toward rCK-2.

	Trial 1	**Trial 2**	**Trial 3**	**Average**	**Std Dev**	**Std error**
Cell+PTX (100ng/ml)+CK2 (500ng/ml)	58	18	51	**42**	21.4	12.3
CK2 (500ng/ml)	294	482	798	**525**	254.7	147.0
β_2M (500ng/ml)	66	134	84	**95**	35.2	20.3
ZAS	1025	424	980	**810**	334.8	193.3
PBS	75	29	73	**59**	26.0	15.0

(d) Migration of RTS-11 toward CK-2. The RTS-11 cells were used right after they were split.

	Number of cells migrated toward CK-2
CK2 (10ug/ml)	61
β_2M (10ug/ml)	43
CK2 (1ug/ml)	346
β_2M (1ug/ml)	47
CK2 (500ng/ml)	231
β_2M (500 ng/ml)	92
ZAS in PBS (10%)	617
PBS alone	105

(e) Migration of RTS-11 toward CK-2. The RTS-11 cells were plated overnight after they were split.

	Number of cells migrated toward CK-2
CK2 (10ug/ml)	too clumpy to count
β_2M (10ug/ml)	93
CK2 (1ug/ml)	too clumpy to count
β_2M (1ug/ml)	87
CK2 (500ng/ml)	324
β_2M (500ng/ml)	52
ZAS	274
PBS	93

Table B3: Raw data of the densitometry analysis for the transcript expression of CK-2/CK-2.1 *in vivo*. The expression was detected by RT-PCR. The ratios were derived by dividing the band intensity of CK/CK-2.1 by the band intensity of EF1-α. The transcript expression pattern of HK, Spleen, and Liver is presented here.

Brain

		EF1-alpha	CK2/CK2.1	Ratio (Gene of interest/ internal control)
0hr	CK-2	815	199	0.244
	CK-2.1	815	337	0.413
4hr	CK-2	503	13	0.026
	CK-2.1	503	106	0.211
8hr	CK-2	983	89	0.091
	CK-2.1	983	168	0.171
24hr	CK-2	854	22	0.026
	CK-2.1	854	49	0.057

HK

		EF1-alpha	CK2/CK2.1	Ratio (Gene of interest/ Internal control)
0hr	CK-2	464	232	0.500
	CK-2.1	464	186	0.401
4hr	CK-2	825	95	0.115
	CK-2.1	825	134	0.162
8hr	CK-2	795	54	0.068
	CK-2.1	795	81	0.102
24hr	CK-2	798	42	0.053
	CK-2.1	798	13	0.016

Spleen (group 1)

		EF1-alpha	CK2/CK2.1	Ratio (Gene of interest/ Internal control)
0hr	CK-2	630	291	0.462
	CK-2.1	630	211	0.335
4hr	CK-2	726	427	0.588
	CK-2.1	726	45	0.062
8hr	CK-2	628	246	0.392
	CK-2.1	628	20	0.032
24hr	CK-2	601	115	0.191
	CK-2.1	601	5	0.008

Spleen (group 2)

		EF1-alpha	CK2/CK2.1	Ratio (Gene of interest/ Internal control)
0hr	CK-2	883	153	0.173
	CK-2.1	883	0	0.000
4hr	CK-2	878	310	0.353
	CK-2.1	878	0	0.000
8hr	CK-2	952	0	0.000
	CK-2.1	952	0	0.000
24hr	CK-2	1061	0	0.000
	CK-2.1	1061	0	0.000

Table B4: Raw data of the densitometry analysis for the transcript expression of CK-2/CK-2.1 *in vitro*. The expression was detected by RT-PCR. The ratios were derived by dividing the band intensity of CK-2/CK-2.1 by the band intensity of EF1-α. The transcript expression pattern of stimulated primary cultured rainbow trout head kidney leukocytes and peripheral blood leukocytes is presented here. In the homozygous fish (CK2/CK2.1), the bold letter denotes the values associated with that specific allele. C stands for control, whereas P stands for PHA stimulated.

(a) Densitometry analysis results for HKLs.

	EF1-alpha	**CK2/CK2.1**	**Ratio**	**Average**	**Std. Dev**	**Std. Error**
CK2-C	718	710	0.989	**0.669**	0.452	0.319
	620	217	0.35			
CK2-P	429	805	1.88	**1.68**	0.28	0.198
	503	745	1.48			
CK2.1-C	684	560	0.819	**0.446**	0.527	0.373
	466	34	0.073			
CK2.1-P	472	536	1.14	**1.03**	0.15	0.106
	607	560	0.923			
CK2/**CK2.1-C**	351	118	0.336	**0.211**	0.178	0.126
	515	44	0.085			
CK2/**CK2.1-P**	732	766	1.05	**0.71**	0.478	0.046
	674	250	0.371			
CK2-C/CK2.1	351	213	0.607	**0.561**	0.065	0.338
	515	265	0.515			
CK2-P/CK2.1	732	509	0.70	**0.88**	0.265	0.187
	674	718	1.07			

(a) Densitometry analysis results for PBLs.

	EF1-alpha	**CK2/CK2.1**	**Ratio**	**Average**	**Std. Dev**	**Std. Error**
CK2-C	1643	1815	1.1	0.705	0.559	0.395
	3544	1094	0.31			
CK2-P	1486	1892	1.27	0.96	0.438	0.31
	2170	1412	0.65			
CK2.1-C	1532	1332	0.87	0.695	0.247	0.175
	2672	1396	0.52			
CK2.1-P	1446	1805	1.25	0.92	0.467	0.33
	2393	1413	0.59			
CK2/**CK2.1-C**	1432	322	0.22	0.685	0.658	0.465
	1636	1878	1.15			
CK2/**CK2.1-P**	1525	810	0.53	0.915	0.544	0.385
	1748	2269	1.3			
CK2-C/CK2.1	1432	688	0.48	0.64	0.226	0.16
	1636	1310	0.8			
CK2-P/CK2.1	1525	1586	1.04	0.995	0.064	0.045
	1748	1655	0.95			

Appendix C

Figures

Figure C1: An alignment of the derived amino acid sequences of CK-2 with CK-2.1. Dashes indicate gaps whereas stars indicate identity with the CK2 sequences.

```
CK2             MVTCGTLVKIWTLAVVIAALGWTGTVDAEKLVSCCKTVSRTEVNDPITGYWIQNYNAPCV 60
CK2.1           MVTCGTLVKIWTLAVVIAALGWTGTVDAEKLVSCCKTVSRTEVNDPITGYWIQNYNAPCV 60
                ************************************************************

CK2             RAVIFETKKGLFCSYHKQPWVRRKIHQFEMARLSSTFLSLSIPNSLTSTSTPTTTSLPSS 120
CK2.1           RAVIFETKKGLFCSYHKQPWVRRKIHQFEMARLSSTFLSLSIPNSLTSTSTPTTTSLPSS 120
                ************************************************************

CK2             PP----------------------------------------SSLFPLSSSSPSVPSS 138
CK2.1           PPSINSSPPSLTSTSTSLTSSPSSSPPSLTSTPTFLPSSPLSVFSSLFPLSSSSPSVPSS 180
                **                                          ***************

CK2             PPSLSSSPSHLLSSLFPASSSPPSISSSPPSFSSPRHWESTKNASTQQSTSNQ 191
CK2.1           PPSLSSSPSHLLSSLFPASSSPPSISSSPPSFSSPRHRESTKNASTQQSTSNQ 233
                ****************************************** *****************
```

Figure C2: ELISA assay to determine the titre of the rabbit anti-CK2 produced in rabbit 2. About 10ml rabbit blood was collected before each injection and used to perform ELISA. Serum was separated form erythrocytes by allowing the blood sample to coagulate. ELISA plates were coated with 1μg r-CK2. After blocking, the plates were probed with primary antibody (rabbit serum) with several dilutions. Following incubation, the secondary antibody (anti-rabbit AP-conjugated)) was added to a final dilution of 1:5000. After each successive injection the titre increased gradually.

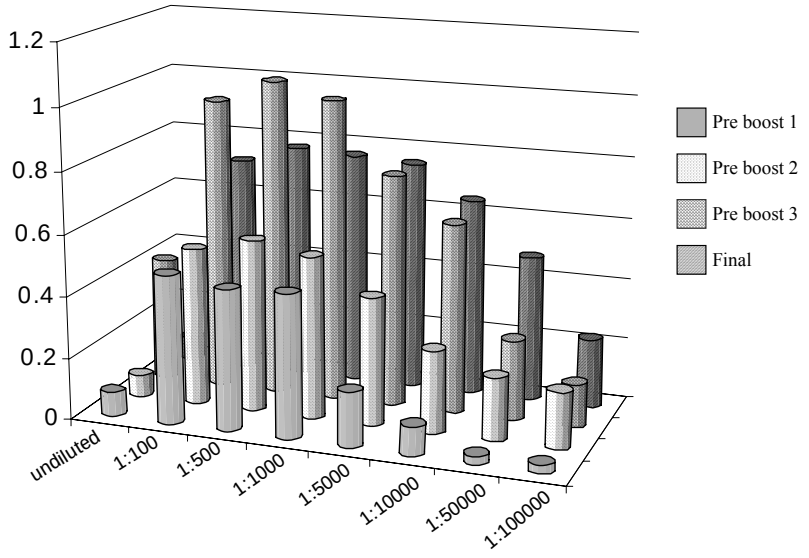
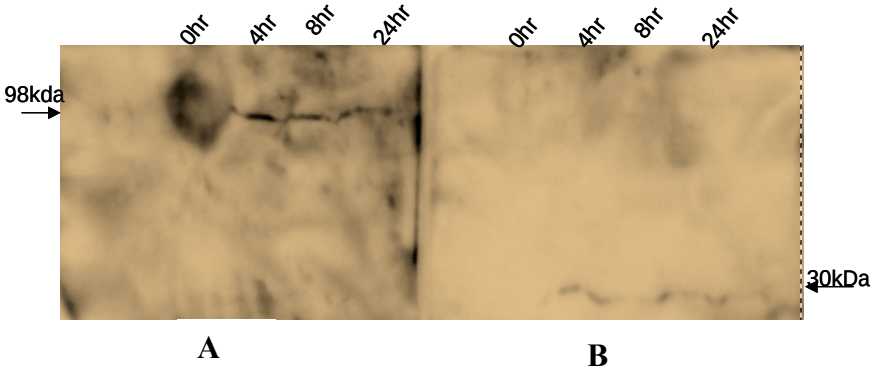


Figure C3: Western blot of brain tissues extracts. A time course experiment was carried out by injecting the rainbow trout with PHA and sacrificing them after 4hr, 8hr, and 24hr. Protein extract from the brain tissue was used to perform western blot analysis. (A) Western blot, which was probed with purified anti-CK-2 antibody at a final dilution of 1:50. (B) A second blot that was probed with antibody specific toward chemokine domain.



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