Characterization of Casein Kinase I-γ2, as a Serine/Threonine Protein Kinase Associated with the Adaptor Protein, Nck

Nadia Cardillo Marricco

Department of Medicine, Division of Experimental Medicine
McGill University, Montreal
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Abstract

CKI-γ2 is a serine/threonine protein kinase of unknown biological function. This study is the first to detect CKI-γ2, as mRNA and protein, widely expressed in mouse tissues and mammalian cells. In addition, CKI-γ2 as a protein of 75kDa was immunologically detected coprecipitating with Nck, an adaptor protein involved in receptor tyrosine kinase (RTK) signaling. CKI-γ2 coprecipitated with Nck presents similar enzymatic properties as the recombinant and endogenous CKI-γ2, demonstrating that the immunoreactive p75 CKI-γ2 associated with Nck is indeed CKI-γ2. These results suggest that Nck-mediated signaling downstream of activated RTKs could involve CKI-γ2 as an important component. The finding that Nck is a substrate of CKI-γ2, suggests that the function of Nck may be regulated by CKI-γ2. Furthermore, observations that insulin negatively regulates p75 CKI-γ2 activity provide evidence for a role of the Nck-CKI-γ2 complex in insulin signaling. Understanding the insulin regulation of CKI-γ2 may point to the biological function of this enzyme.

Résumé

CKI-γ2 est une sérine/thréonine protéine kinase dont le rôle cellulaire nous est encore inconnu. Cette étude est la première à montrer que CKI-γ2 est largement exprimée dans plusieurs tissus chez la souris et dans plusieurs lignées cellulaires. CKI-γ2 est détectée comme une protéine de 75kDa et nous avons démontré qu'elle coimmunoprecipite avec la protéine adaptrice Nck. Cette dernière est impliquée dans la transmission cellulaire des signaux initiés par l'activation de récepteurs membranaires de type tyrosine kinase. CKI-γ2 associée à Nck, présente des propriétés enzymatiques identiques à celles observées pour la protéine CKI-γ2 d'origine endogène ou recombinante, confirmant ainsi son identité. Ces résultats nous révèlent que CKI-γ2 est un nouvel élément des processus de signalisation initiés par l'activation de récepteurs membranaires. De plus, nous avons observé que la protéine Nck est phosphorylée par CKI-γ2 et que l'insuline induit une inhibition temporaire de l'activité de cet enzyme. Ces résultats suggèrent que les fonctions de Nck peuvent être modulées par cet enzyme et que le complexe Nck-CKI-γ2 peut participer à la transmission des signaux initiés par le récepteur de l'insuline. Une meilleure compréhension des mécanismes impliqués dans la régulation de l'activité de la protéine CKI-γ2 par l'insuline nous permettra d'éclaircir son rôle biologique.

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Chapter 1

Introduction

1.1 Casein Kinases

Casein Kinases are serine/threonine (ser/thr) protein kinases with a preference for acidic substrates such as casein and phosvitin. Their nomenclature is derived from the substrate used for their purification and characterization, namely casein. The Casein Kinases described here differ from the mammary gland membrane-bound enzyme, which phosphorylates secreted milk proteins such as casein (1).

Casein Kinases are ubiquitous enzymes widely expressed within the animal and plant kingdom, and in lower eukaryotes (reviewed in 2, 3). Furthermore, Casein Kinases are distributed in several subcellular compartments such as the cytosol, membranes, nuclei, ribosomes, and mitochondria. Since casein is not present in most of the cell types where Casein Kinases are expressed, other proteins must serve as substrates. Indeed, Casein Kinases phosphorylate several components of the DNA replication, transcription, and translation machinery, as well as metabolic enzymes and regulatory proteins. The wide variety of substrates for Casein Kinases suggests the importance of these multipotential protein kinases in regulation of cellular functions.

Unlike many other protein kinases, Casein Kinases are unique in that they exist in an already active state under most physiological conditions (3). The activity of these enzymes is not sensitive to Ca^{2+}, calmodulin, or phospholipids (2). In addition, regulation of their activities, such as by hormones, second messengers, or targeting to specific intracellular sites, cannot be clearly demonstrated. Although Casein Kinases were first identified many years ago, their study has not been aggressively pursued over the years. The lack of dramatic effect on the function of proteins phosphorylated by Casein Kinases and the absence of known regulatory mechanisms for these enzymes have made them very difficult to study.

1.2 Casein Kinase I and II

Casein Kinases consist of two main activities, designated Casein Kinase I (CKI) and Casein Kinase II (CKII), based on their elution profile from chromatography purification on diethylaminoethylcellulose (DEAE-cellulose) (4). CKI elutes first at low ionic strength, while CKII elutes at higher salt concentrations. Furthermore, CKI and CKII differ by several parameters. From their amino acid (aa) sequence, CKI and CKII are two distinct protein kinases, no more related to each other than to other protein kinases. CKI are monomeric enzymes with molecular weight (Mr) in the range of 30-42kDa (reviewed in 2, 3). In contrast, CKII is a tetramer composed of different subunits, $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ (2-7). The α subunits have Mr values of 37-44kDa, while the β subunit is smaller with Mr values of 24-26kDa (reviewed in 2, 3). The α subunits possess the catalytic activity, whereas the β subunit acts as a regulator of the α subunit's catalytic activity (8-11).

Both Casein Kinases require Mg^{2+}, in the range of 5-15mM, for optimal activity (2). However, one of the characteristics for CKI is an absolute specificity for ATP as a phosphate donor. The CKI Km values for ATP, range from 7- 20μM (3). In contrast, CKII utilizes GTP (Km: 5-100μM) almost as effectively as ATP (Km: 5-30μM) for the phosphotransferase reaction (3).

CKI and CKII phosphorylate common substrates, but each type of Casein Kinase uses different recognition sequences and therefore modifies different sites on the same protein. The phosphorylation sequence requirements for each Casein Kinase were determined using genetic variants of casein as exogenous substrates. CKI prefers serine residues with a cluster of acidic amino acids, such as glutamic acid (Glu), on the amino-terminal (N-terminal) side of the phosphorylated serine, while CKII phosphorylates serine or threonine with a cluster of acidic residues on the carboxyl-terminal (C-terminal) side (2). Phosphorylated serine (Ser(P)) also appears to function as an acidic determinant for both CKI and CKII, since dephosphorylated casein leads to significantly lower rates of phosphorylation by

either enzyme (12-14).

Efforts to determine the role of Casein Kinases have led to a search for potential regulators of these enzymes. Activators of CKII are highly charged basic compounds such as polyamines, spermine, spermidine, polylysine, histones, and protamine (reviewed in 3). Inhibitors of CKII are acidic compounds such as heparin, 2,3-diphosphoglycerate, and inositol hexasulfate (reviewed in 2). Inhibitors of CKII do not inhibit CKI, with the exception of inositol hexasulfate, which at high concentrations also inhibits CKI. The isoquinoline sulfonamide compound, N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide (CKI-7), at low concentrations selectively inhibits CKI with a half-maximal inhibition (IC_{50}) of 9.5μM compared to 90μM for CKII (15). CKI-7 inhibits CKI competitively with respect to ATP and serves as a useful tool to study CKI. To date, no physiological activators of CKI have been identified.

1.3 Casein Kinase I

1.3.1 Consensus Phosphorylation Motifs

More sophisticated studies have identified consensus motifs for phosphorylation by CKI. From a degenerate peptide library, it was determined that CKI strongly selects peptides with a negatively charged residue, such as aspartic acid (Asp), at the –3 position to the phosphorylated serine (16). The peptide $(Asp)_4$-Xaa-Xaa-Ser (where Xaa may be any amino acid) is a specific substrate for CKI and is useful in routine assays for this enzyme (17). The importance of a prior phosphorylated serine (Ser(P)) in the CKI consensus motif, Ser(P)-Xaa-Xaa-Ser/Thr, was also confirmed in studies using synthetic peptides based on the sequence of casein (18) and SV40 large T antigen (19), the latter recognized as an *in vivo* CKI substrate.

1.3.2 CKI Substrates

CKI phosphorylates several proteins implicated in protein translation, most of which bind directly to RNA. These substrates include the translational initiation factors, eIF-4B and eIF-5 (20), and a 95kDa protein in messenger ribonucleoprotein (mRNP) particles (21). In addition, CKI-dependent phosphorylation of the 82kDa subunit of eIF-2B seems to be required for eIF-2B guanine nucleotide exchange activity (22). CKI also phosphorylates four aminoacyl-tRNA synthetases altering their binding to tRNA-sepharose and inhibiting tRNA aminoacylation (23).

Several DNA-binding proteins involved in DNA replication or transcription are phosphorylated by CKI, including RNA polymerase-I and -II (RNA Pol-I or -II) (24). Although *in vitro*, the activity of RNA Pol-II is not affected by CKI phosphorylation (24, 25), in Novikoff ascites tumor cells, RNA Pol-II activity is stimulated 5-7 fold upon its phosphorylation by CKI (26). CKI phosphorylates SV40 large T-antigen on physiological sites and inhibits T-antigen mediated initiation of viral DNA replication (27, 28). The p53 tumor-suppressor protein is also phosphorylated by CKI *in vitro* and *in vivo* (29, 30), yet mutation of these CKI sites has no effect on p53 transcription activity (31). Lastly, CKI phosphorylates the transcription factor, cAMP response element modulator (CREM), and results in enhanced DNA binding activity (32).

Several regulatory proteins are phosphorylated by CKI including the insulin receptor (33), p75 Tumor Necrosis Factor (TNF) receptor (34, 35), m3-muscarinic receptor (36), and β-Platelet Derived growth factor (PDGF) receptor (37). In general, CKI phosphorylation of these receptors negatively regulates their activity. Intracellular regulatory proteins such as phosphatase inhibitor-2 (the negative regulatory subunit of protein phosphatase-1 (PP1)) (38) and DARPP-32 (the dopamine-and cAMP-regulated phosphoprotein inhibitor of PP1) (39, 40) are phosphorylated by CKI. Phosphorylation by CKI prevents the normal inactivation of these PP1 regulators, maintaining PP1 inactivated.

Substrates of CKI include enzymes involved in metabolism such as acetyl CoA carboxylase (41, 42), the enzyme that catalyses the initial step of fatty acid synthesis. Although phosphorylation by CKI has no direct effect on acetyl CoA carboxylase activity, it is proposed that CKI may play an indirect role in the insulin-mediated activation of this enzyme (43). Glycogen synthase (GS), the rate-limiting enzyme in glycogen synthesis, is phosphorylated by CKI, and this results in decreased activity of GS (44-48). In addition, when GS is previously phosphorylated by the cAMP-dependent protein kinase (PKA), the rate of phosphorylation and thereby inactivation by CKI is increased by 3-to 5- fold (49, 50).

CKI phosphorylates many proteins that play an important role in maintenance of cell shape and morphology. For example, CKI found in the cytosol and membrane of erythrocytes, phosphorylates red cell membrane proteins such as spectrin (51, 52), a major determinant of erythrocyte shape and deformability. CKI also phosphorylates the sialoglycophorins (51) and fibrinogen (53, 54). Lastly, CKI phosphorylates troponin, myosin light chain, myosin light chain kinase, tubulin, and microtubule-associated protein 2, components of mammalian muscles (55, 56).

Evidence *in vivo* for protein phosphorylation by CKI is very sparse and includes glycogen synthase (57), SV40 large T-antigen (27), CREM (32), and p53 (29). Although a variety of protein substrates have been reported for CKI, in relatively few cases have functional changes as a result of phosphorylation been noted.

1.4 Yeast CKI

In budding yeast, *Saccharomyces cerevisiae*, HRR25, YCK1, YCK2, and YCK3, have been identified as CKI proteins with molecular weights ranging from 55 to 62kDa. (58, 59, 60, 61).

YCK1 and YCK2 (also called CK1 and CK2, respectively) are an essential gene pair, whereby deletion of either gene alone has no effect on growth but deletion of both genes results in inviability of yeast cells (59). The two protein kinases share an overall 77% aa identity to each other, and 94% identity in their kinase domains alone (60). The predicted protein sequences of the YCK genes define for larger CKI enzymes of 60-62kDa that possess 50-55% aa identity with their mammalian counterparts (62). The Yck proteins perform functions essential for cell growth and division (60). More specifically, the phenotype of YCK mutant cells suggests that the Yck proteins play a role in bud morphogenesis, possibly in the control of cell growth polarity and cytokinesis. In addition , overexpression of YCK1 and YCK2 confers halotolerance to yeast cells (59). The C-terminal sequences of the Yck proteins includes a prenylation motif that anchors them to the cytoplasmic side of the plasma membrane and is required for their full biological function (60, 62, 63).

The third CKI in *S. cerevisiae*, HRR25, encodes for a 58kDa gene product, whose kinase domain shows 86% similarity and 68% aa identity with other mammalian CKI enzymes (64). The HRR25 gene was first identified from a yeast mutant cell line sensitive to DNA-damaging agents (58). Phenotypic analysis of HRR25 mutants suggested that HRR25 encodes a regulator of DNA double strand-break repair. In addition, HRR25 mutant cells cannot enter meiosis, and during mitosis, show defects in nuclear segregation. HRR25 is also involved in transcriptional response to DNA damage through its interaction with the phosphorylated transcription factor, Swi6 (65). Whereas Yck proteins associate exclusively with the plasma membrane, most hrr25 protein localizes with the cell nucleus and contains a putative nuclear localization signal (58).

The fourth CKI gene in budding yeast, is the YCK3 gene (61). Yck3 protein is plasma membrane-associated but most cofractionates with nucleus, like Hrr25p. Genetic studies showed that YCK3 and HRR25 constitute an essential gene pair, although Yck3p can weakly substitute for Yck1p-Yck2p. YCK1, 2, and 3 genes in

increased dosages can overcome the growth defect of gcs1 gene Δ mutant cells, a gene which normally mediates the resumption of cell proliferation from the starved, stationary-phase state (61).

In fission yeast, *Schizosaccharomyces pombe*, there are four CKI-like genes, *cki1*$^+$, *cki2*$^+$, *hhp1*$^+$, and *hhp2*$^+$, which encode proteins from 45 to 50kDa in size (66, 67, 68).

cki1$^+$ and *cki2*$^+$ gene products are most related structurally (67-69% aa identity in the catalytic domain) and enzymatically (complement yckΔ mutants) to the YCK gene products of budding yeast (66). Subcellular fractionation experiments demonstrate that cki1p and cki2p are both cytoplasmic proteins. Gene disruption experiments show that neither *cki1*$^+$ nor *cki2*$^+$ is essential for cell viability. Overexpression of cki2p leads to severe growth defects and aberrant morphology, suggesting that *cki2*$^+$ regulates cell morphology.

hhp1$^+$ and *hhp2*$^+$ are closely related to each other (75% aa identity in kinase domains) and to mammalian CKI-α (>68% aa identity) (68). Furthermore *hhp*$^+$ gene products share >65% aa identity with Hrr25p of *S. cerevisiae*, complementing HRR25Δ mutants. Single and double mutants in *hhp*$^+$ genes are sensitive to DNA-damaging agents that cause DNA double strand breaks, suggesting that *hhp1*$^+$ and *hhp2*$^+$ regulate DNA repair pathways.

Although the gene structure and classical characterization of CKI activity revealed CKI enzymes are most closely related to ser/thr protein kinase, several of the yeast CKI (HRR25, *cki1*$^+$, *hhp1*$^+$, and *hhp2*$^+$) have been identified as dual-specificity enzymes (69). The enzymes expressed in *Escherichia coli* (*E. Coli*), were recognized by anti-phosphotyrosine antibodies and subsequently shown by phosphoamino acid analysis to phosphorylate serine, threonine, and tyrosine residues. The function of tyrosine phosphorylation by CKI enzymes is not known but may point to an important function of these enzymes in yeast. Nevertheless,

in yeast, CKI enzymes seem to function in regulation of DNA repair and cell morphology.

1.5 CKI Mammalian Isoforms

Originally considered a single entity, in recent years it has become apparent that casein kinase I consists of multiple isoforms that are more closely related to each other (>50% identity) than to any other protein kinase (≤ 21% identity) (70). Mammalian CKI activity stems from the existence of 7 closely related isoforms, designated CKI-α, CKI-β, CKI-γ1-3, CKI-δ, and CKI-ε. All together, mammalian CKI isoforms comprise a distinct family of protein kinases.

1.5.1 CKIα and CKIβ

CKI-α and CKI-β full-length cDNAs were first isolated from a bovine brain cDNA library (70). The coding sequence of the CKIα cDNA encoded for a 325 amino acid protein with predicted molecular weight of 37.6kDa, whereas CKI-β cDNA predicted for a 336 amino acid protein of 38.7kDa. By Northern blot analysis, the messages for CKI-α were 4.1 and 2.2 kb in bovine brain total RNA but only the 2.2 kb transcript was found in bovine thymus total RNA. Isolation of two complete cDNAs (CKI-α and CKI-β), likely representing 2 distinct genes, constituted the first findings that CKI activity in mammalian tissues or cell extracts was composed of more than one protein kinase activity. Furthermore, in the same study, a partial cDNA of CKI-α that contained a 84-base pair (bp) in-frame shift, was isolated from the bovine brain cDNA library, and designated CKI-αL. This suggested the existence of an alternatively spliced form of the CKI-α gene product. In a subsequent study, characterization of genomic DNA flanking the exon unique to CKI-αL confirmed that CKI-α and CKI-αL are two proteins that arise by alternative splicing of a common pre-mRNA molecule transcribed from a single gene (71). The full-length cDNA encoding the CKI-α isoform was also cloned from a rabbit skeletal muscle cDNA library (72). The recombinant CKI-α expressed in *E. Coli* is an active kinase that phosphorylates α-

casein, phosvitin, and a CKI-specific peptide, DDDDVASLPGLRRR (D4). The enzyme was inhibited by CKI-7 with an IC_{50} of 70 μM. Surprisingly heparin inhibited phosphorylation of phosvitin or the D4 peptide, while polylysine stimulated phosphorylation of the D4 peptide. The fact that CKI-α activity was altered by treatment with two known regulators of CKII activity, made it clear that the individual CKI isoforms have different enzymatic properties and hence could have distinct cellular functions.

Lastly, the human homolog of CKI-α (hCKI-α) was cloned by PCR using primers designed from sequence of *Xenopus laevis* CKI cDNA (73). The human CKI-α cDNA encoded for a 38.9kDa and 337 amino acid protein, that is identical to the bovine CKI-α protein except for an additional 12 amino acids at the C-terminus. The human CKI-α gene was mapped to human chromosome 13q13 by fluorescent in situ hybridization (FISH). This study represented the first cloning and chromosomal localization of a gene coding for a human CKI.

1.5.2 CKIγ Subfamily

In the same study by Rowles *et al.*, a partial cDNA from the bovine brain cDNA library was isolated and encoded for a third CKI enzyme, designated CKI-γ (70). Using this bovine partial CKI-γ cDNA as a probe, a rat testis cDNA library was screened (74). Three full-length cDNAs, designated CKI-γ1, CKI-γ2, and CKI-γ3, were isolated and encoded for proteins of 390, 414, and 448 amino acids, respectively. Their respective predicted molecular weights are 43, 45.5, and 49.7kDa. All three CKI-γ enzymes contain signature CKI sequences and share 51-59% identity to other mammalian CKI isoforms. Nevertheless, the γ-enzymes share 90-93% identity within their protein kinase domains and 69-78% identity over their entire length, and therefore form a novel subfamily of CKI. The messages for CKI-γ1 (2 kb), CKI-γ2 (1.5 and 2.4 kb), and CKI-γ3 (2.8 kb) were detected by Northern analysis of poly(A)+ RNA from rat testis using isoform specific probes based on 3′ non-coding sequences. All three CKI-γ isoforms are

active enzymes in *E. Coli* and autophosphorylate in the presence of ATP and Mg^{2+}. Heparin stimulated the phosphorylation of the D4 peptide by all three enzymes. However, CKI phosphorylation of phosvitin and α-casein by all three γ-isoforms is inhibited by heparin. CKI-7 inhibited all three CKI-γs, although less effectively than for other mammalian CKI isoforms. The IC_{50} values for CKI-7 inhibition of CKI-γ1 and -γ2 activity, were 200μM and 60μM, respectively. For CKI-γ3, 30% inhibition was achieved with 300μM CKI-7.

The human homolog of CKI-γ2 (hCKI-γ2) was isolated from a human testis cDNA library (75). The hCKI-γ2 cDNA predicted for a 416 amino acid protein with 83% homology at the nucleotide level and 94% homology at the aa level with the rat CKI-γ2. The 3′ region of human CKI-γ2 cDNA (30% coding sequences and 70% 3′UTR) was used as a probe for Northern blot analysis of various human tissues poly (A)+ RNA. A CKI-γ2 mRNA of 2.4 kb was found in testis poly (A)+ RNA but a weaker 3 kb transcript was found in all tissues. The human CKI-γ2 mRNA of 2.4 kb agrees with the length of rat CKI-γ2 transcript reported by Zhai *et al.*(74), and indicates that the expression of CKI-γ2 in both humans and rats, is highest in testis. The human CKI-γ2 gene, designated CSNK1G2, was mapped to chromosome 19 p13.3 by FISH and PCR analysis of human/rodent hybrid cell panels.

1.5.3 CKIδ and CKIε

Rowles *et al.*, also isolated a PCR product from the bovine brain cDNA library, that encoded for partial sequences of yet another CKI enzyme, designated CKI-δ (70). At the same time, another group independently isolated a partial CKI-δ PCR product from rabbit testis (59). Screening of a rat testis cDNA library with this second PCR product yielded the full length CKI-δ cDNA, encoding a protein of 428 amino acids and molecular weight of 49kDa (76). Northern blot analysis of rabbit or rat total RNA, using a probe generated from the coding region of CKI-δ, revealed 3 hybridizing species of 3.5-4.1, 2.2, and 1.9kb. The largest transcript

was detected in all tissues examined whereas the 2.2 and 1.9kb messages are only found in testis. The enzyme was expressed in *E. Coli* as an active kinase, with an apparent molecular weight of 55kDa, which phosphorylates α-casein, phosvitin, and the D4 peptide. The recombinant enzyme was inhibited by heparin when phosvitin and α-casein were used as substrates, with half-maximal inhibition at 11.5μg/ml and 200μg/ml of heparin, respectively. When the D4 peptide was used, recombinant CKI-δ was also inhibited by CKI-7 with an IC_{50} of 12μM. In contrast, heparin activated CKI-δ activity towards the D4 peptide by 4- to 5-fold and with half-maximal activation at 9.5μg/ml. Truncation of 111 amino acids from the C-terminus of CKI-δ, resulted in an enzyme that was no longer activated by heparin, suggesting that the C-terminus is required for the effect of heparin. This was the first evidence for the existence of a regulatory domain in a CKI enzyme. Nevertheless, inhibition by CKI-7 was relatively unchanged with IC_{50} of 10μM and 12μM for the truncated CKI-δ and full length CKI-δ, respectively.

Based on the sequence of bovine CKI-δ, a novel human CKI enzyme, designated hCKI-ε, was cloned from a human placental cDNA library (77). The cDNA for hCKI-ε predicts for a 416 amino acid protein with a molecular mass of approximately 47.3kDa. The hCKI-ε catalytic domain (285aa) is 53-98% identical to the kinase domain of other mammalian CKIs but is most closely related to CKI-δ isoform in that it has an extended C-terminal tail (97% identical in their kinase domains and 53% identical in C-terminus extensions) (76, 77). However, hCKI-ε is a distinct gene from CKI-δ isoform for several reasons. Most notably, hCKI-ε and hCKI-δ were mapped by FISH to distinct loci, that is chromosome 22q12-13 and chromosome 17q25, respectively (77, 78). Northern blot analysis of poly (A)+ RNA from HeLa cells and of total RNA from 8 different human cell lines gave one major message of 2.7-2.9kb and a second minor message of 1.6-1.77kb, for hCKI-ε. hCKI-ε expressed in *E. Coli* is an active enzyme able to autophosphorylate and phosphorylate the D4 peptide, phosvitin, and α-casein. Phosphorylation of the D4 peptide by the recombinant

hCKI-ε is inhibited by CKI-7, with IC_{50} of 18μM.

Despite the identification of numerous CKI isoforms in mammals, we still do not understand what is the cellular function of CKI.

1.6 Potential Roles of CKI

Information from lower eukaryotes such as yeast has facilitated the study of mammalian CKI isoforms and allowed the elucidation of possible CKI functions. The CKI gene family can be divided into three subgroups according to their structure and function (67).

The first subgroup including CKI enzymes that regulate nuclear processes, are mostly localized in the nucleus, and appear involved in response to DNA damage, especially in the repair of DNA double-strand breaks. Members of the nuclear subfamily include HRR25 which was cloned in a screen for budding yeast mutants sensitive to DNA double strand breaks (58, 63, 64), and the *S. pombe* essential gene pair, *hhp1*$^+$ and *hhp2*$^+$ (67, 68). The mammalian homologs of these genes are the closely related CKI-δ and CKI-ε also implicated in response to DNA damage. For instance, the human CKI-ε gene partially complements *S. cerevisiae* with a deletion of the HRR25 gene (77).

The second subgroup includes the inner plasma membrane-associated YCK gene products in budding yeast, which regulate cytoplasmic processes such as cell morphology and cell growth polarity (62, 63, 66). Overexpression of YCK genes results in halotolerance suggesting CKI functions in osmolarity signaling pathway in budding.yeast (79). Other members include their fission yeast counterparts, *cki1*$^+$ and *cki2*$^+$ (66), and the mammalian CKI-γ isoforms (63). Although CKI-γ1 and CKI-γ3 are not associated with the plasma membrane, they are considered part of this subgroup because they restored cell morphology and growth defects in YCKΔ mutant yeast cells (74). The third γ-isoform, CKI-γ2, was not tested for its ability to rescue YCKΔ mutant yeast cells.

The third subgroup includes CKI-α and -β which are found in the nucleus and cytoplasm and may thus perform multiple functions. Despite their high sequence homology to HRR25 (70% aa identity in kinase domains), these enzymes are considered a separate CKI subgroup due to their different structure and function (67). Mammalian CKI-α exhibits cell cycle-dependent localization to mitotic spindles suggesting its function in mitosis (80), a role supported by the requirement of CKI-α for proper cell cycle progression from interphase to mitosis in the fertilized mouse oocyte (81). In addition, a preparation of CKI-α from human erythrocytes possessed tyrosine kinase activity, and catalyzed the tyrosine phosphorylation of a variety of substrates such as angiotensin-II, tyrosine-containing peptides, alkylated bovine serum albumin (BSA), band 3, and ankyrin (82). Furthermore, partially purified preparations of mammalian CKI-α are activated by insulin, interleukin-1, and, TNF, suggesting a role of CKI enzymes in signal transduction (83, 84, 85).

Lastly, cell-surface receptor activity and signaling have been shown to be regulated by CKI phosphorylation. For instance, serine phosphorylation of the p75 TNF receptor by CKI, negatively regulates TNF-induced apoptosis (34, 35). The phospholipase C-coupled m3-muscarinic receptor, a G-protein-coupled receptor (GPCR), is serine phosphorylated in an agonist-sensitive manner by CKI-α (36). Similarly, phosphorylation of Ste2p, a yeast GPCR, by CKI enhances internalization and downregulation of Ste2p (86). Finally, in our laboratory we have demonstrated that serine phosphorylation of the ligand-activated β-PDGFR by CKI-γ2 inhibits the receptor's autophosphorylating activity (37).

1.7 CKI Structure and Regulation

Studies of yeast and mammalian CKI isoforms have enabled us to learn more of the structure of CKI family members. All CKI enzymes consist of a highly conserved approximately 290 aa N-terminal kinase domain followed by a poorly conserved C-terminal region that is highly variable in length (24->200 aa) and

amino acids (63, 67, 87, 88). With the exception of mammalian CKI-α and CKI-β, all mammalian CKI isoforms and their yeast counterparts in *S. cerevisiae* and *S. pombe*, show C-terminal extensions beyond their kinase domains. It has become apparent that several functions can be attributed to the C-terminus extensions of CKI family members. For instance, the C-terminus is important in some isoforms for the proper targeting and localization of CKI proteins in the cell. Both Yck proteins from *S. cerevisiae* contain C-terminal consensus sequence motif for prenylation which is the principal feature responsible for targeting Yck proteins to the plasma membrane, and in absence of this prenylation motif, Yck proteins do not have full biological function as they are inappropriately localized in the cells. Secondly, the C-terminal extensions of various CKI isoforms are implicated in autoregulation of CKI catalytic activity. Studies with synthetic substrates and α-casein, indicate that autophosphorylation of CKI in its C-terminus, can inhibit its kinase activity (89, 76). Kinetic characterization of the purified catalytic domain and other C-terminal deletion mutants of *S. pombe* Cki1 protein suggests that the C-terminal domain regulates the catalytic activity (89). For instance, autophosphorylation in the C-terminal domain of Cki1p results in a 4-fold decrease in affinity for substrates. In contrast, C-terminal truncated Cki1p presents a 3-fold activation in catalytic rate. This activation may arise from the removal of an inhibitory domain present in the intact enzyme. Similarly, for CKI-δ, its C-terminal domain has also been demonstrated to regulate the catalytic activity. In fact, a truncated CKI-δ lacking the C-terminal 111aa, was no longer activated by heparin (76). A phosphorylation-dependent inhibitory domain in CKI-δ was mapped to a 26aa sequence in its C-terminal tail, whereby activation by heparin or phosphatases appears to be dependent on the presence of C-terminal domain (87). A similar mechanism of regulation by the C-terminus was identified for CKI-ε (88). CKI-ε contains a hyperphosphorylated C-terminal tail that inhibits its activity towards exogenous substrates but may be relieved by phosphatase treatment and intracellular proteolysis *in vivo*. The existence of such a mechanism of regulation *in vivo* was confirmed with observations that although CKI-δ and CKI-ε are able to autophosphorylate *in vivo*, they are actively

maintained in cells in dephosphorylated, active state by serine/threonine phosphatases (90). This dynamic autophosphorylation/dephosphorylation cycle regulated by phosphatases, provides a first clear mechanism for CKI regulation *in vivo*.

Other mechanisms of regulation are proposed for CKI although they are not clearly demonstrated. Briefly, hormonal stimulation (83, 91, 92) or viral transformation (93) may regulate CKI activity as insulin-treated and virally-transformed cells have elevated CKI activity (83, 93). Lastly, inhibition of CKI activity by phosphatidylinositol 4,5 bisphosphate (PIP2) was observed (94, 95) whereby interaction with a substrate-bearing organelle such as the plasma membrane in this case, may provide another means of regulation.

1.8 CKI-γ2 and Nck

In our laboratory, a previous study (96) has reported that in the yeast two-hybrid system, the extremity C-terminal of CKI-γ2 interacted with a particular signal transducing molecule called, Nck.

Nck is a 47kDa protein, localized in the cytoplasm and nucleus (97) of cells. Both Nck mRNA and Nck protein are detected in a wide variety of tissues and cell lines, indicating that Nck has a fundamental role for cell activity (98, 99). Nck cDNA was first isolated from a human melanoma library by cross-hybridizing with a monoclonal antibody produced against the melanoma-associated antigen, MUC18 (100, 101). However, Nck cDNA encodes for a 377 amino acid protein that has no homology with MUC18, and lacks any signal peptide, transmembrane region, or catalytic domain. Instead, Nck consists almost exclusively of Src homology (SH) domains (101), which are conserved protein modules that mediate specific protein-protein interactions in signal transduction cascades downstream of activated cell surface receptors. Nck amino acid sequence reveals three consecutive SH3 domains at the N-terminus followed by one SH2 domain at the C-terminus (101). The SH2 domain (reviewed in 102, 103) is a sequence of

approximately 100aa that recognizes specific phosphotyrosine sequences (104). The shorter SH3 domains are approximately 60aa long (reviewed in 102, 103) and recognize short proline-rich motifs in consensus PXXP sequence (105). Nck is part of a special group of non-enzymatic signaling molecules which includes Grb2, Shc, Crk, and p85 subunit of PI3-K. They are collectively known as adaptors and function to link signaling molecules within the cells (102, 106).

Binding of growth factors (GF) by receptor tyrosine kinases induces receptor dimerization and autophosphorylation of specific tyrosine residues within their intracellular domains. Nck is believed to act as an adaptor protein that couples activated receptor tyrosine kinases (RTK) via its SH2 domain to downstream proline-rich effector proteins via its SH3 domains. A variety of extracellular stimuli, including EGF, PDGF, NGF, insulin, high affinity IgE R (FcεRI) clustering, as well as activation of T- and B-cell antigen receptor all lead to increased phosphorylation of Nck (107, 108, 98, 109, 99). Indeed, Nck via its SH2 domain, binds directly to the PDGF receptor (110), the EphB1 receptor (111), and indirectly with the EGF receptor (112), in a ligand-dependent manner. In addition, Nck binds via its SH2 domain, to non receptor protein tyrosine kinases (NRTK) such as $p60^{src}$ and $p56^{lck}$, members of the Src family of protein kinases (107, 113). Nck also binds to the intracellular tyrosine-phosphorylated IRS-1 (108), which upon insulin stimulation, associates the insulin receptor, and serves as a docking protein for SH2-containing signaling molecules. The association of Nck with RTKs, NRTKs, and other intracellular tyrosine-phosphorylated proteins suggests that Nck is a common mediator of signal transduction. Finally, Nck is oncogenic whereby its overexpression leads to cellular transformation of NIH 3T3 fibroblasts, colony formation in soft agar, and tumor formation in nude mice (98, 107).

The biological responses involving Nck are sparse. Nck has been implicated in photoreceptor axonal guidance in *Drosophila* (114), and dorsoventral patterning in *Xenopus* (115). Nck is also implicated in mitogenesis as it is required for

PDGF-induced DNA synthesis in fibroblasts (116). Furthermore, Nck overexpression causes continuous proliferation of PC12 (Rat adrenal pheochromocytoma) cells and blocks their NGF- and bFGF-induced differentiation (117).

Despite these evidences of Nck involvement in specific biological responses, little is known about the mechanism by which Nck mediates these processes. More specifically, what are the signaling pathways initiated downstream of Nck recruitment? In recent years, identification of effector molecules that interact with Nck's SH3 domains has been viewed as a step to understanding more about Nck. Nck through its SH3 domains, has been shown to interact with effector proteins implicated in the regulation of cytoskeleton by small GTPases (Ras, Rho, Rac, and Cdc42) (118). For example, Nck interacts with SOS, an activator of Ras (119), and with the Wiskott Aldrich syndrome protein (WASP), a putative effector of Cdc42 (120, 121). In addition, several serine/threonine protein kinases including, PRK2 (similar to the Rho effector PKN) (122), NIK (a Ste20 family member coupled to activation of MEKK4 and JNK) (123), and PAK (a Ste20 family member that is activated by Cdc42 and Rac) (124, 125, 126) were shown to associate with Nck through its SH3 domains. Lastly, Nck association with Cbl, a major RTK substrate that possibly inhibits cellular signaling (127-129), and Sam68, an RNA binding protein that is a major substrate of c-Src in mitotic cells (97), confirms its place downstream of RTK and NRTK but does not explain the biological significance of these interactions.

Recently, CKI-γ2 has been added to the list of effector proteins that associate to the SH3 domains of Nck. In fact, a previous study in our laboratory (96), demonstrated that the C-terminal domain of CKI-γ2 interacted with the three SH3 domains of Nck in the yeast two-hybrid system. The presence of a proline-rich motif in the C-terminus of CKI-γ2 supports its interaction with the SH3 domains of Nck. Furthermore, a serine/threonine protein kinase activity with CKI enzymatic characteristics was observed in Nck immunoprecipitates and associated

with recombinant Nck constructs encoding its SH3 domains. CKI-γ2 and Nck interaction was shown to be a direct and constitutive association that was unaltered by insulin stimulation. In addition, a rabbit polyclonal antibody raised against the C-terminal domain of CKI-γ2 (from the yeast two-hybrid screen) recognized a p75 CKI-γ2 in the Nck immunoprecipitates from HTC-IR (rat transformed hepatocytes overexpressing the insulin receptor) cells. Overall, these data support the *in vitro* and *in vivo* interaction of CKI-γ2 with the adaptor protein, Nck.

1.9 Rationale and Objectives of Study

The molecular weight of CKI-γ2 predicted from the cDNA (45.5kDa) is smaller than that of CKI-γ2 coprecipitated with Nck (approximately 75kDa). It is important then to characterize this CKI-γ2-like p75 coprecipitated with Nck in order to demonstrate that it is indeed a CKI enzyme. In addition, possible explanations for this molecular weight difference, such as hyperphosphorylation or post-translational modification of the enzyme, will be explored. Confirming the association of CKI-γ2 with Nck, a mediator of signal transduction from activated RTKs, will greatly enhance our knowledge of the different signaling pathways in which Casein Kinases participate. It is therefore worthwhile to determine if Nck is a substrate of CKI-γ2, in order to understand the effects of casein kinase phosphorylation events in cells. Regulation is still an open question for the casein kinases, and therefore determining if extracellular stimuli can modulate CKI-γ2 activity associated with Nck is significant. For instance, since Nck is recruited by IRS-1 to the insulin signaling pathway, it would be worthwhile to see if CKI-γ2 activity is modulated by insulin. In summary, the objectives of this study are to characterize the p75 CKI-γ2 constitutively associated with Nck. Secondly, it will be determined if Nck is phosphorylated by CKI-γ2. In addition, it will be determined whether insulin is a possible extracellular stimuli that modulates CKI-γ2 activity and consequently Nck phosphorylation *in vivo*.

Chapter 2

Materials and Methods

2.1 Cloning of the Full-Length Mouse CKI-γ2 cDNA

A cDNA fragment coding for the C-terminal region of the mouse CKI-γ2 cDNA (residues192-414), previously isolated from a yeast two-hybrid screen using the three SH3 domains of Nck as a bait (96), was used to probe a mouse brain cDNA library (Sratagene) (approximately 1.8×10^6 independent recombinants). The probe was labeled by random priming and hybridized to filters containing approximately 1×10^5 recombinants per filter. Prehybridization was performed at 63°C, for at least 2h, in a 5 × Denhardt's solution (1 × Denhardt's solution: 0.02% (w/v) each of Ficoll, polyvinylpyrrolidone, and BSA), 6 × SSC (1 × SSC: 0.15M sodium chloride and 15mM sodium citrate, pH 7.0), 0.01M EDTA pH 7.4, 0.5% SDS, and 0.1mg/ml of salmon sperm DNA. Hybridization was performed overnight under identical conditions to those used for prehybridization except for addition of the radiolabeled probe 0.5-1 $\times 10^6$ cpm/ml (instead of salmon sperm DNA). Nitrocellulose filters were washed twice in 2 × SSC containing 0.1% SDS for 30 min at 63°C. If necessary, a third wash was carried out in 0.2 × SSC containing 0.1% SDS for 30-60 min at 63°C. Following autoradiography of the dried filters using intensifying screens, 8 positive clones were identified and plague-purified. Each cDNA insert was rescued in pBluescript by co-infection with a helper phage according to the manufacturer's instructions (Sratagene). The cDNAs were sequenced on both strands using the dideoxy method of Sanger *et al.*, (130, 131).

2.2 Antibodies

A polyclonal CKI-γ2 antibody (1602) was produced by immunizing rabbits with a GST fusion protein comprising the C-terminal portion of CKI-γ2 (residues 192-414) (96). A second polyclonal CKI-γ2 antibody (1986) was produced by immunizing rabbits with a GST fusion protein comprising the full-length CKI-γ2

(residues 1-414). A polyclonal Nck antibody (1698) was previously raised in rabbits by immunizing with a GST fusion protein containing the three SH3 domains of human Nck (residues 1-251) (96). The specificity of each antibody was confirmed by displacement with their respective antigen. A second polyclonal Nck antibody raised against the SH3 domains of human Nck (residues 1-254) (UBI#06-288) was purchased from Upstate Biotechnology Inc.. Recombinant anti-phosphotyrosine antibody coupled to horseradish peroxidase (RC20) was purchased from Transduction Laboratories.

2.3 Cell Culture

All cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C and in the presence of 1× solution of antibiotics-antimycotic (100 × solution: 10 000U/ml penicillin G sodium, 10 000μg/ml streptomycin sulfate, 25μg/ml amphotericin B as Fungizone ® in 0.85% saline; Life Technologies, Inc.). HTC-IR (rat hepatoma transformed cells overexpressing the human insulin receptor) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% fetal bovine serum (FBS, Life Technologies) and maintained in selection medium containing 40μg/ml of Geneticin (G418, Life Technologies). Rat-2 (rat fibroblasts), 293 (adenovirus Ad5 transformed human embryonal kidney), and Cos-1β (monkey kidney) cells were grown in DMEM containing 10% FBS. PC12 (rat adrenal pheochromocytoma) cells were grown in DMEM containing 10% FBS, 5% horse serum (Life Technologies) and maintained in selection medium containing 40μg/ml of G418. FAO (rat hepatoma transformed cells derived from H35) (132) cells were grown in F-12 COON's Modification (Sigma) containing 10% FBS. HeLa (human cervix epitheloid carcinoma) cells were grown in Minimum Essential Medium (Life Technologies) containing 10% FBS. 3T3-L1 fibroblasts (mouse embryo fibroblasts) were grown in DMEM low glucose (1000mg/ml) containing 10% calf serum (Life Technologies) and 2 days post-confluency were treated with differentiation medium comprised of DMEM high glucose (4500mg/ml) containing 10% FBS, 1μg/ml insulin, 0.1μg/ml dexamethasone, and 112μg/ml

isobuthylmethylxanthene. After 2 days, the differentiation medium is replaced by DMEM high glucose containing 10% FBS and 1μg/ml insulin for 2 more days. The medium is changed every 2 days for DMEM high glucose containing 10% FBS. The 3T3-L1 adipocytes (mouse embryo adipocytes) are used 10-12 days after the beginning of the differentiation process.

2.4 Cell Lysis, Immunoprecipitation, and Immunoblotting

Preparation of cell lysates-HTC-IR cells were washed twice with cold phosphate-buffered saline (PBS: 0.08M dibasic sodium phosphate, 0.02M monobasic sodium phosphate, and 0.1M sodium chloride; pH 7.5) and then lysed in 1%Triton lysis buffer (50mM Hepes pH 7.5, 150mM Sodium Chloride, 10% (v/v) Glycerol, 1% (v/v) Triton-X-100, 1.5mM Magnesium Chloride, 10mM Sodium Pyrophosphate, 10mM Sodium Fluoride, 1mM Phenylmethylsulfonyl Fluoride, 10μg/ml Aprotinin, 10μg/ml Leupeptin) for 10min at 4°C with gentle agitation. Lysates were clarified by centrifugation at 10 000 × g for 10 min at 4°C in JA-17 rotor (Beckman). In some experiments, cells were lysed in homogenization buffer (5mM Tris-HCl pH 7.4, 1mM Benzamidine, 1mM Phenylmethylsulfonyl Fluoride, 2mM Sodium Fluoride, 2mM Sodium Orthovanadate, 1mM Magnesium Chloride, and 0.25M Sucrose) and homogenized using a Teflon-glass homogenizer. Lysates were clarified by centrifugation at 10 000 × g for 10 min at 4°C in JA-17 rotor (Beckman).

Immunoprecipitation-For CKI-γ2 immunoprecipitation, clarified cell lysates were incubated with normal serum and either protein A- or protein G-sepharose beads for 1h at 4°C. The precleared lysates were then incubated with anti-CKI-γ2 antibodies and either protein A- or protein G-sepharose beads for 90min, 4h, or overnight at 4°C with gentle agitation. For Nck immunoprecipitation, clarified cell lysates were incubated with normal serum and protein A-sepharose beads for 1h at 4°C. The precleared cell lysates were subsequently incubated with anti-Nck antibodies and protein A-sepharose beads for 90 min, 4h, or overnight at 4°C with gentle agitation. Immunocomplexes were washed twice with 0.1% Triton lysis

buffer (20mM Hepes pH 7.5, 150mM NaCl, 0.1% Triton X-100, 10% (v/v) Glycerol, 1mM Phenylmethylsulfonyl Fluoride, 10μg/ml Aprotinin, 10μg/ml Leupeptin) and eluted in Laemmli buffer (133).

Immunoblotting-Upon SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to nitrocellulose or polyvinyldifluoride (PVDF) membrane, and blocked in TBS-T (TBS plus 0.05% (v/v) Tween 20) containing 5% of dry milk for 30min at room temperature. Incubation with the primary antibody was carried out for 1h at room temperature followed by extensive washes in TBS (0.02 M Tris base and 0.1 M sodium chloride; pH 7.6) and TBS-T. Incubation with the secondary antibody (protein A conjugated to horseradish peroxidase, abbreviated protein A-HRP) in TBS-T was carried out for 1h at room temperature. After extensive washes in TBS and TBS-T, the proteins were visualized by enhanced chemiluminescence (ECL, Amersham Corp.). In some experiments, incubation with the secondary antibody (goat anti-rabbit antibodies labeled with ^{125}I, abbreviated ^{125}I-GAR) was carried out in TBS-T containing 5% milk for 1h at room temperature. Following extensive washes with TBS and TBS-T, the membrane was dried and proteins visualized by autoradiography.

2.5 Characterization of Anti-CKI-γ2 Antibodies

Clarified HTC-IR cell lysates prepared in homogenization buffer were incubated with anti-CKI-γ2 antibody (1602) or normal serum in the presence of Protein A-Sepharose beads, for 90min at 4°C. Immunoprecipitated proteins and proteins from total cell lysate (20μg) were resolved by SDS-PAGE (7.5% acrylamide gel) and transferred to nitrocellulose. CKI-γ2 was revealed by immunoblotting with anti-CKI-γ2 antibody (1602), protein A-HRP and ECL.

Similarly, clarified HTC-IR cell lysates prepared in 1% Triton lysis buffer were incubated with anti-CKI-γ2 antibody (1986) or normal serum in the presence of protein A-Sepharose beads, for 4h at 4°C. Immunoprecipitated proteins and proteins from total cell lysate (20μg) were resolved by SDS-PAGE (7.5%

acrylamide gel) and transferred to nitrocellulose. CKI-γ2 was revealed by immunoblotting with anti-CKI-γ2 antibody (1986), protein A-HRP, and ECL.

2.6 Reciprocal Coimmunoprecipitation of CKI-γ2 and Nck

Clarified HTC-IR cell lysates prepared in 1% Triton lysis buffer were precleared with the normal serum and protein A-Sepharose beads. Precleared lysates were subsequently immunoprecipitated with anti-Nck antibody (UBI#06-288) and protein A-Sepharose beads, for overnight at 4°C. The immunoprecipitated proteins and proteins form total cell lysate were resolved by SDS-PAGE (7.5% acrylamide gel) and transferred to nitrocellulose. CKI-γ2 was revealed by immunoblotting with anti-CKI-γ2 antibody (1602), protein A-HRP, and ECL. The blot was stripped (30min at 65°C in a solution containing 62.5mM Tris-HCl, pH 6.7, 1% SDS, and 0.1M β-mercaptoethanol) and probed for Nck by immunoblotting with anti-Nck antibody (1698), protein A-HRP, and ECL.

In the reciprocal experiment, clarified HTC-IR cell lysates prepared in 1% Triton lysis buffer were precleared with the normal serum and protein G-Sepharose beads. Precleared lysates were subsequently immunoprecipitated with anti-CKI-γ2 antibody (1602) and protein G-Sepharose beads for overnight at 4°C. The immunoprecipitated proteins and proteins from total cell lysate were resolved by SDS-PAGE (7.5% acrylamide gel) and transferred to nitrocellulose. Nck was revealed by immunoblotting with anti-Nck antibody (1698), Protein A-HRP, and ECL. The blot was stripped and probed for CKI-γ2 by immunoblotting with anti-CKI-γ2 antibody (1602), protein A-HRP, and ECL.

2.7 Northern Blot Analysis of CKI-γ2 and Nck

Total RNA was prepared from mouse tissues (adipocyte, adrenals, brain, heart, kidney, liver, lung, muscle, and testis) by using guanidium isothiocyanate for the cesium chloride purification of RNA from tissues, originally described by

Chirgwin *et al.*, (134). Total RNA was prepared from mouse (3T3-L1 fibroblasts, 3T3-L1 adipocytes), rat (HTC-IR, Rat-2, PC12, FAO), and human (HeLa) cell lines by using a single-step guanidium thiocyanate-phenol-chloroform method for isolation of RNA from cultured cells, originally described by Chomczynski and Sacchi (135).

Equal amounts of total RNA (10μg/lane) were size fractionated by electrophoresis in 1% agarose-0.2.M formaldehyde gel, transferred onto a Hybond-N nylon membrane (Amersham Life Technologies) by capillary action in 20 × SSC. The nylon membrane was rinsed in 6 × SSC and UV cross-linked to covalently immobilize the RNA to the membrane. Prehybridization of the nylon membrane was performed for minimum of 1h at 65°C in a solution of 10 × Denhardt's solution, 10% Dextran sulphate, 50mM Tris-HCl pH 7.5, 1M sodium chloride, 0.1% sodium pyrophosphate, 1% SDS, and 0.1mg/ml of salmon sperm DNA. Hybridization was performed overnight at 65°C with the same solution used for prehybridization except for the addition of the radiolabeled probe ~1 × 10^6 cpm/ml (instead of salmon sperm DNA). Following hybridization, the membrane was washed three times with 2 × SSC, and 0.1% SDS for 20min at room temperature and once for 20min at hybridization temperature of 65°C. The membrane was then subjected to autoradiography at -80°C with intensifying screens.

Double-stranded cDNA probes from mouse CKI-γ2 and human Nck coding sequences were labeled by random priming with [α^{32}P]-dCTP using the T7 Quick Prime Kit (Pharmacia Biotechnologies). The probes used were as following: for CKI-γ2, an approximately 300-bp *XmnI*/*Eco*RI fragment (nucleotides 927-1242) from the carboxyl-terminal domain of CKI-γ2 coding sequence that is more specific to the γ2-isoform of CKI; for Nck, an approximately 750-bp *Bam*HI/*Eco*RI fragment (nucleotides 1-753), from the three SH3 domains of Nck coding sequence.

2.8 Western Blot Analysis of CKI-2 and Nck

Adipocyte, brain, heart, kidney, liver, lung, muscle, and testis tissues were prepared from Balb/c mice. Approximately 1g of each mouse tissue was adjusted to a final concentration of 15% in homogenization buffer and homogenized with a Polytron PT10-35 homogenizer. Homogenates were prepared by centrifugation at 258 000 × g for 30min at 4°C in a SW40Ti rotor (Beckman). The resulting supernatants (100μg of protein per tissue) were resuspended in Laemmli buffer, boiled for 2-3min, resolved by SDS-PAGE (7.5% acrylamide gel), and then transferred to nitrocellulose. CKI-γ2 or Nck was detected by immunoblotting with anti-CKI-γ2 antibody (1986) or anti-Nck antibody (1698), respectively, followed by ^{125}I-GAR and autoradiography.

2.9 Plasmids and Constructs

For bacterial expression as glutathione S-transferase (GST) fusion proteins, cDNAs encoding for full-length mouse CKI-γ2, full-length human Nck, and isolated SH domains of human Nck were amplified by polymerase chain reaction (PCR) using specific oligonucleotides with the *Bam*HI/*Eco*RI linkers to facilitate subcloning into pGEX4T2 or pGEX2TK plasmids (Pharmacia Biotech Inc.). For mammalian expression, the full-length coding CKI-γ2 cDNA, excised from CKI-γ2 pGEX4T2 by *Bam*HI/*Eco*RI digestion, was subcloned into pCDNA3.1+ (Invitrogen). All PCR-generated fragments were sequenced by dideoxynucleotide method.

2.10 Preparation of GST-Fusion Proteins

GST-fusion protein comprising the full-length CKI-γ2 (GST-CKIγ2, residues 1-414) and various SH domains of Nck (GST-SH3#1, residues 1-65; GST-SH3#2, residues 108-165; GST-SH3#3, residues 188-266; GST-3×SH3, residues 1-251; GST-SH2, residues 282-377; GST-Nck, full-length coding sequence, residues 1-377) were expressed in *E. Coli*. The proteins were isolated using glutathione-agarose beads according to the manufacturer's instructions (Pharmacia) and gave essentially single bands on Coomassie Blue-stained SDS-polyacrylamide gels,

with the exception of GST-CKIγ2, GST-Nck and GST-3×SH3 which contained degraded smaller fragments.

In some experiments, GST-CKIγ2 immobilized on glutathione-agarose beads (designated, GST-CKIγ2 on beads) was eluted from the beads according to the supplier's recommendations, concentrated on Centricon-30 (Amicon, Inc.) and finally resuspended in buffer A (50mM Tris-HCl pH 7.5, 1mM EDTA, 1mM EGTA, 50mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 2mM benzamidine, 10μg/ml aprotinin and 10μg/ml leupeptin) (designated, eluted GST-CKIγ2).

2.11 GST-CKIγ2 *In Vitro* Kinase Assay

The kinase activity of GST-CKIγ2 on beads and of eluted GST-CKIγ2 was verified by an *in vitro* kinase assay of the recombinant enzyme alone or in the presence of an exogenous substrate, α-casein (15μg, Sigma). The kinase reactions were carried out in 25μl of the Kinase Buffer (75mM Tris-HCl pH 7.5, 6mM Magnesium Acetate, 1mM EDTA, 0.4mM EGTA and 1mM β-mercaptoethanol) (74). After a preincubation of 5min at 30°C, kinase reactions were initiated by the adding 20μM adenosine triphosphate (ATP, Sigma) and 10μCi of [γ^{32}P]-ATP (DuPont NEN). The reactions were stopped after 20 min at 30°C by the addition of 5μl of 6 × Laemmli buffer followed by boiling for 2-3 min. The samples were subjected to SDS-PAGE (12% acrylamide gel) and autoradiography.

The enzymatic characteristics of recombinant CKI-γ2 kinase activity were analyzed by the *in vitro* phosphorylation of α-casein (5μg) using 30ng of eluted GST-CKIγ2. In experiments where ion dependence was determined, 0, 1, 2.5, 5, or 10mM magnesium chloride ($MgCl_2$) or manganese chloride ($MnCl_2$) was added to the assay containing ion-free Kinase Buffer. In experiments where the substrate specificity was determined, 5μg of myelin basic protein (MBP, Sigma), 15μg of α-casein, 15μg of β-casein (Sigma), 15μg of phosvitin (Sigma) or 15μg

of PolyGluTyr (Sigma) was added to the assay. In experiments where the effect of N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide (CKI-7, Seikagaku Corp.) was determined, 0, 25, 50, or 100μM of CKI-7 was added to the assay.

2.12 CKI-γ2 Immunoprecipitation and *In Vitro* Kinase Assays

Clarified HTC-IR cell lysates prepared in 1% Triton lysis buffer were submitted to CKI-γ2 immunoprecipitation for 4h at 4°C, using anti-CKI-γ2 antibody (1986) and protein A-Sepharose beads. Washes of the immunocomplexes with 0.1% Triton lysis buffer was followed by one wash with Kinase Buffer. The enzymatic characteristics of endogenous CKI-γ2 kinase were determined by an *in vitro* kinase assay of the CKIγ2 immunoprecipitates in the presence of α-casein (5μg). In experiments where ion dependence was determined, 0, 1, 2.5, 5, or 10mM $MgCl_2$ or $MnCl_2$ was added to the assay containing ion-free Kinase Buffer. In experiments where the substrate specificity was determined, 5μg MBP, 15μg α-casein, 15μg β-casein, 15μg phosvitin, or 15μg PolyGluTyr was added to the assay. In experiments where the effect of CKI-7 was determined, 0, 25, 50, or 100μM of CKI-7 was added to the assay.

2.13 Nck Immunoprecipitation and *In Vitro* Kinase Assays

Clarified HTC-IR cell lysates prepared in 1% Triton lysis buffer were submitted to Nck immunoprecipitation for 90 min at 4°C, using anti-Nck antibody (1698) and protein A-Sepharose beads. Washes of the immunocomplexes with 0.1% Triton lysis buffer was followed by one wash with Kinase Buffer (20mM Hepes, pH 7.5, 1mM dithiothreitol, 5mM magnesium chloride, 10mM β-glycerophosphate). The enzymatic characteristics of the kinase activities associated with endogenous Nck were determined by an *in vitro* kinase assay on Nck immunoprecipitates in presence of α-casein (5μg). In experiments where ion dependence was determined, 0, 1, 2.5, 5, or 10mM $MgCl_2$ or $MnCl_2$ was added to the assay containing ion-free Kinase Buffer. In experiments where the substrate specificity was determined, 5μg MBP, 15μg α-casein, 15μg β-casein, 15μg

phosvitin, or 15μg PolyGluTyr was added to the assay. In experiments where the effect of CKI-7 was determined, 0, 25, 50, or 100μM of CKI-7 was added to the assay.

2.14 CKI-γ2 Dual-Specific Kinase Assay

CKI-γ2 was assayed *in vitro* for dual specific kinase activity, by analyzing the autophosphorylating activity of 0.0125, 0.025, 0.05, 0.1, 0.2, or 0.5μg of eluted GST-CKIγ2. The *in vitro* kinase assay was carried out as indicated above except that it was carried out in the presence of nonradioactive ATP only. After separation by SDS-PAGE (7.5% acrylamide gel), proteins were transferred to nitrocellulose, and the membrane was blocked in blocking buffer (1% bovine serum albumin in 10mM Tris pH 7.5, 100mM sodium chloride, and 0.1% Tween 20) for 20min at 37°C. The autophosphorylation on tyrosine residues of GST-CKIγ2 was detected using the anti-phosphotyrosine antibody (RC20) horseradish peroxidase conjugated, and ECL. A sample of EGF stimulated A431 (human epidermoid carcinoma) cell lysates provided with the RC20 antibody was used as a positive control.

2.15 Phosphatase Treatment of Immunoprecipitated CKI-γ2

Clarified HTC-IR cell lysates prepared in 1% Triton lysis buffer were immunoprecipitated for CKI-γ2 using anti-CKI-γ2 antibody (1602) and protein A-sepharose beads for 4h at 4°C. CKI-γ2 immunoprecipitates were washed four times with either Potato Acid Phosphatase (PAP) Buffer (40mM PIPES pH 6.1, 1mM DTT, 20μg/ml aprotinin, and 20μg/ml leupeptin) or Protein Phosphatase 2A (PP2A) Buffer (20mM HEPES pH 7.0, 1mM DTT, 1mM $MnCl_2$, 100μg/ml BSA, and 50μM leupeptin). CKI-γ2 immunoprecipitates were finally resuspended in 100μl of either PAP buffer or PP2A buffer. The protein precipitates were submitted to dephosphorylation with PAP (6U/ml) (Boehringer Mannheim) or with PP2A catalytic subunit (20mU/ml) (Boehringer Mannheim) for 30min at 37°C. Dephosphorylated CKI-γ2 immunoprecipitates were washed five times

with 0.1% Triton lysis buffer, resuspended in Laemmli buffer, and boiled for 2-3min. Samples were resolved by SDS-PAGE (7.5% acrylamide gel), transferred to nitrocellulose, and immunoblotting was performed using anti-CKI-γ2 antibody (1602), protein A-HRP, and ECL

2.16 In Vitro Transcription and Translation of CKI-γ2

In vitro transcription/translation-The pCDNA3.1+ vector containing the CKI-γ2 cDNA was linearized with *Eco*RI (cutting at the 3′ end of the insert). The linearized DNA was purified by phenol:chloroform extraction, and ethanol precipitation. *In vitro* transcription (from the T7 promoter) and translation (in the presence of [^{35}S]-methionine/cysteine (Trans ^{35}S-label, ICN)) of 1μg of the pcDNA3.1+-derived plasmid containing CKI-γ2 was carried out using the TNT T7 coupled rabbit reticulocyte system (Promega) according the manufacturer's instructions. *In vitro* transcription and translation using the TNT T7 coupled wheat germ extract system (Promega) was also performed. The *in vitro* transcription and translation of CKI-γ2 was carried out for 15, 30, 60, or 90min. Samples of *in vitro*-translation products were resuspended in Laemmli buffer, and boiled for 2-3min. After separation by SDS-PAGE (7.5% acrylamide gel), the gel was prepared for fluorography using EN^{3}HANCE (DuPont) and then exposed for autoradiography.

Immunoprecipitation with anti-CKI-γ2 antibody-[^{35}S]-labeled *in vitro*-translated CKI-γ2 was incubated with anti-CKI-γ2 antibody (1986) or normal serum in the presence of protein A-Sepharose beads, for 4h at 4°C. The immunoprecipitated proteins were washed twice with PBS, resuspended in Laemmli buffer, and boiled for 2-3min. After separation by SDS-PAGE (7.5% acrylamide gel), the gel was prepared for fluorography using EN^{3}HANCE and then exposed for autoradiography.

In vitro kinase assay-[^{35}S]-labeled *in vitro*-translated CKI-γ2 was

immunoprecipitated with anti-CKI-γ2 antibody (1986), washed twice with cold PBS, and once with CKIγ Kinase Buffer before being submitted to an *in vitro* kinase assay in the presence or absence of α-casein (5μg) as described above.

2.17 Transient Transfections

Lipofectamine-mediated transient transfections of 293 and Cos-1β cells were performed according to the manufacturer's protocol (Life Technologies). Briefly, 293 and Cos-1β cells (2.5×10^5 cells) were plated in six-well tissue culture plates 18-24h prior to transfection. The transfection start was when 0.5, 1, 2, 3, and 5μg of CKI-γ2 pCDNA3.1+ DNA and 10μl of LipofectAMINE reagent were added to each plate in 1ml of serum-free medium (OPTI-MEM I Reduced Serum Medium, Life Technologies). After 16 h, 1ml of DMEM containing 20% FBS was added. After 8h (24h after start of transfection), the medium was replaced with fresh DMEM containing 10% FBS. Cells were harvested 48-72h after the start of transfection. The cells were washed two times with PBS, and lysed in boiling Laemmli buffer. The cell lysates was recovered, passed several times through a needle to shear DNA, and boiled for 2-3min. The proteins in the total cell lysates were resolved by SDS-PAGE (7.5% acrylamide gel) and transferred to nitrocellulose. Transient transfections of CKI-γ2 pcDNA 3.1+ were analyzed by immunoblotting with anti-CKI-γ2 antibody (1986), ^{125}I-GAR, and autoradiography.

2.18 *In Vitro* Phosphorylation of Nck by CKI-γ2

In vitro kinase assays were performed with 1μg of GST-CKIγ2 on beads in the presence of various exogenous substrates. The exogenous substrates include 2μg of GST-SH3#1, GST-SH3#2, GST-SH3#3, GST-3×SH3, GST-SH2, GST-Nck, or GST alone. Similar *in vitro* kinase assays were performed with 1μg of eluted GST-CKIγ2. The samples were subjected to SDS-PAGE (7.5% acrylamide gel), transferred to nitrocellulose, and exposed for autoradiography. Phosphorylated GST-3×SH3 was subjected to proteolytic phosphopeptide mapping (see below).

Proteolytic Peptide Mapping-Phosphopeptide mapping was performed according to the published method of Van Der Geer *et al.*, (136). Briefly, on nitrocellulose membrane, [^{32} P]-labeled proteins were localized by autoradiography. The corresponding bands were excised and soaked in 0.5% polyvinylpyrrolidone (PVP)-360 in 100mM acetic acid for 30min at 37°C. The pieces of membrane were washed five times with water and twice with 50mM ammonium bicarbonate. Tryptic digestion was performed with 10μg TPCK-trypsin (Sigma) in 200μl of 50mM ammonium bicarbonate. Three consecutive tryptic digestions were performed at 37°C (8h incubation, followed by overnight incubation, and lastly a 2h incubation). After centrifugation, the membranes were discarded and the supernatants were lyophilized in a central vacuum concentrator. The dried peptides were oxidized with 50μl of performic acid, diluted in water, and lyophilized again. The samples were finally resuspended in 10μl electrophoresis buffer pH 1.9. Tryptic phosphopeptides were separated by two-dimensional separation on thin-layer cellulose (TLC) plates. In the first dimension, tryptic phosphopeptides were separated by electrophoresis for 30min at 1kV with pH1.9 buffer (formic acid (88% w/v)-acetic acid-water [50:156:1794, vol/vol]). In the second dimension, tryptic phosphopeptides were separated by ascending chromatography in phospho-chromatography buffer (n-butanol-pyridine-acetic acid-water [750:500:150:600, vol/vol]). Visualization of tryptic phosphopeptides was achieved by autoradiography.

2.19 *In Vivo* Phosphorylation of Nck

Subconfluent HTC-IR were serum-starved in DMEM containing 0.5% FBS for 24h prior to the experiment. Cells were washed three times with phosphate-free DMEM containing 0.5% dialyzed FBS (Life Technologies) and then incubated for 4h at 37°C in the same medium containing 0.2mCi/ml of [^{32} P]-orthophosphate (New England Nuclear). After 4h incubation, cells were washed three times in cold PBS and lysed in 1% Triton lysis buffer. Lysates were clarified by centrifugation. [^{32} P]-labeled HTC-IR cell lysates were precleared with normal

serum and protein A-Sepharose beads and subsequently immunoprecipitated for Nck for 4h at 4°C. Nck immunoprecipitates were washed five times with 1% Triton lysis buffer, resuspended in Laemmli buffer, and boiled for 2-3min. The samples were subjected to SDS-PAGE (7.5% acrylamide gel), transferred to nitrocellulose, and exposed for autoradiography. Phosphorylated Nck was subjected to proteolytic phosphopeptide mapping as described above.

2.20 Insulin Regulation of CKI-γ2 Kinase Activity

Cell stimulation and immunoprecipitation-Subconfluent HTC-IR cells were serum-starved in DMEM containing 0.2% FBS for 24h prior to the experiment. Insulin (porcine insulin, Connaught-Novo Laboratories, Willowdale, Canada) was added to HTC-IR cells in culture (final concentration 100nM) for 0, 2, 5, 10, and 30min. At the end of stimulation, basal and insulin-stimulated cells were washed twice with cold PBS and then lysed in 1% Triton lysis buffer. CKI-γ2 was immunoprecipitated with anti-CKI-γ2 antibody (1602) and protein G-sepharose beads for 4h at 4°C. Nck was immunoprecipitated with anti-Nck antibody (1698) and protein A-sepharose beads for 4h at 4°C. The immunoprecipitated proteins were washed twice with 0.1% Triton lysis buffer, resuspended in Laemmli buffer and boiled for 2-3min. The samples were subsequently subjected to *in vitro* gel kinase assays as described below.

In vitro gel kinase assays-α-Casein (50μg/ml) was added to the running polyacrylamide gel solution just prior to polymerization. Immunoprecipitated CKI-γ2 and protein kinase(s) coimmunoprecipitated with Nck were detected directly in the gel, by their ability to phosphorylate the α-casein polymerized in the gel (137). Samples were subjected to SDS-PAGE (7.5% acrylamide gel), and SDS was removed by washing the gels with 20% (v/v) isopropyl alcohol in 50mM imidazole, 28mM iminodiacetic acid, pH 8.0, twice for 60min at room temperature. The gels were then washed with 50mM imidazole, 28mM iminodiacetic acid, pH 8.0, containing 10mM β-mercaptoethanol for 60min at room temperature. Proteins in the gels were then denatured with 8.0M guanidine

HCl in 50mM imidazole, 25mM iminodicetic acid, pH 8.0, containing 50mM β-mercaptoethanol for 90min at room temperature. Protein renaturation was achieved by successive washes at 4° C (2 × 90min, 200ml; overnight, 400ml; 1 × 60min, 200ml) in 25mM imidazole, 14mM iminodiacetic acid, pH 8.0, containing 20mM KCl, 10% sucrose, 10mM β-mercaptoethanol, 1% bovine serum albumin, and 0.04% Tween 20. The gels were equilibrated in a solution containing 10mM HEPES, pH8.0, 10mM β-mercaptoethanol, and 5mM $MgCl_2$ for 60min at room temperature and incubated for an additional 120min following the addition of [γ^{32}P]-ATP (20μCi/ml). Finally, the gels were extensively washed with 5.0% (w/v) trichloroacetic acid containing 1.0% (w/v) sodium pyrophosphate and 1.0% sodium phosphate. After fixing and drying, the gels were exposed for autoradiography.

2.21 Effect of Insulin on *In Vivo* Nck Phosphorylation

Cell labeling, stimulation, immunoprecipitaion, and immunoblotting-Subconfluent HTC-IR cells were labeled with [^{32}P]-orthophosphate as indicated above except that during the last 2, 5, or 30min of labeling, 100nM insulin was added. The clarified HTC-IR cell lysates were precleared with normal serum and subsequently immunoprecipitated for Nck with anti-Nck antibody (1698) and protein A-sepharose beads for 4h at 4°C. One-tenth (1/10) of all Nck immunoprecipitates were subjected to SDS-PAGE (7.5% acrylamide gel), transferred to PVDF membrane, exposed for autoradiography and subjected to phosphoamino acid analysis (see below). Identical experiments were performed with unlabeled HTC-IR cells and the amount of Nck protein in each immunoprecipitate was verified by immunblotting with anti-Nck antibody (1698), protein A-HRP, and ECL.

Phosphoamino Acid Analysis-Phosphoamino acid analysis was performed according to the method of Van Der Geer *et al.*, (136). Briefly, on PVDF membrane, [^{32}P]-labeled protein was localized by autoradiography. The corresponding band was excised and washed 10 times with water. Acid

hydrolysis was performed for 1h at 110°C in 6N HCl. The membranes were then discarded, and after centrifugation, the supernatants were lyophilized and finally resuspended in buffer pH 1.9 containing phosphoserine, phosphothreonine, and phosphotyrosine at 1mg/ml as internal standards. Phosphoamino acids were separated by two dimensional electrophoresis (buffer pH 1.9 and 3.9) on TLC plates, visualized by autoradiography, and identified by comparing to ninhydrin-stained phosphoamino acid standards.

Chapter 3

Results

3.1 Objective #1: Characterization of the immunoreactive p75 CKI-γ2 constitutively associated with Nck.

3.1.1 Cloning of the Full-Length Mouse CKI-γ2 cDNA

A previous study in our laboratory isolated a kinase-related partial cDNA encoding a protein that interacts with the three SH3 domains of Nck in the yeast two-hybrid system and whose amino acid sequence was identical to the C-terminal domain of the rat CKI-γ2 (residues 192-414) (96). In order to obtain the full-length CKI-γ2 cDNA, the CKI-γ2 C-terminal fragment was used as a probe to screen a mouse brain cDNA library. Screening resulted in eight positive signals. All eight clones were taken to plaque purity and completely sequenced. One cDNA, clone #9, was 1336bp long, consisting of 1185bp of coding sequence and 151bp of 3'-untranslated sequence (Fig. 1A). Clone#14 contained a cDNA of 1480bp and consisted of 1185bp of coding sequence and 295bp of 3'-untranslated sequence (Fig. 1A). The deduced amino acid sequence of clone #9 and clone #14 predicted a protein whose amino acid sequence was 99.5% identical to the rat CKI-γ2 (74) but which lacked the first 20 amino acids of the coding sequence. Although the coding regions for clone#9 and clone#14 were identical, their 3'-untranslated sequences diverged immediately after the stop codon. The 3'-untranslated sequence of clone #9 was identical to that published for rat CKI-γ2. The 3'-untranslated sequence of clone#14 was identical to that of the CKI-γ2 C-terminal cDNA fragment isolated from the yeast two-hybrid system, and therefore suggests that it naturally exists.

A third positive clone, clone#2, was 1700bp long and consisted of 78bp of 5'-untranslated sequence and overlapping sequences in CKI-γ2 coding region including the first 20 amino acids missing in clone#9 and #14. However, the

Figure 1: Cloning of Full-Length Mouse CKI-γ2 cDNA

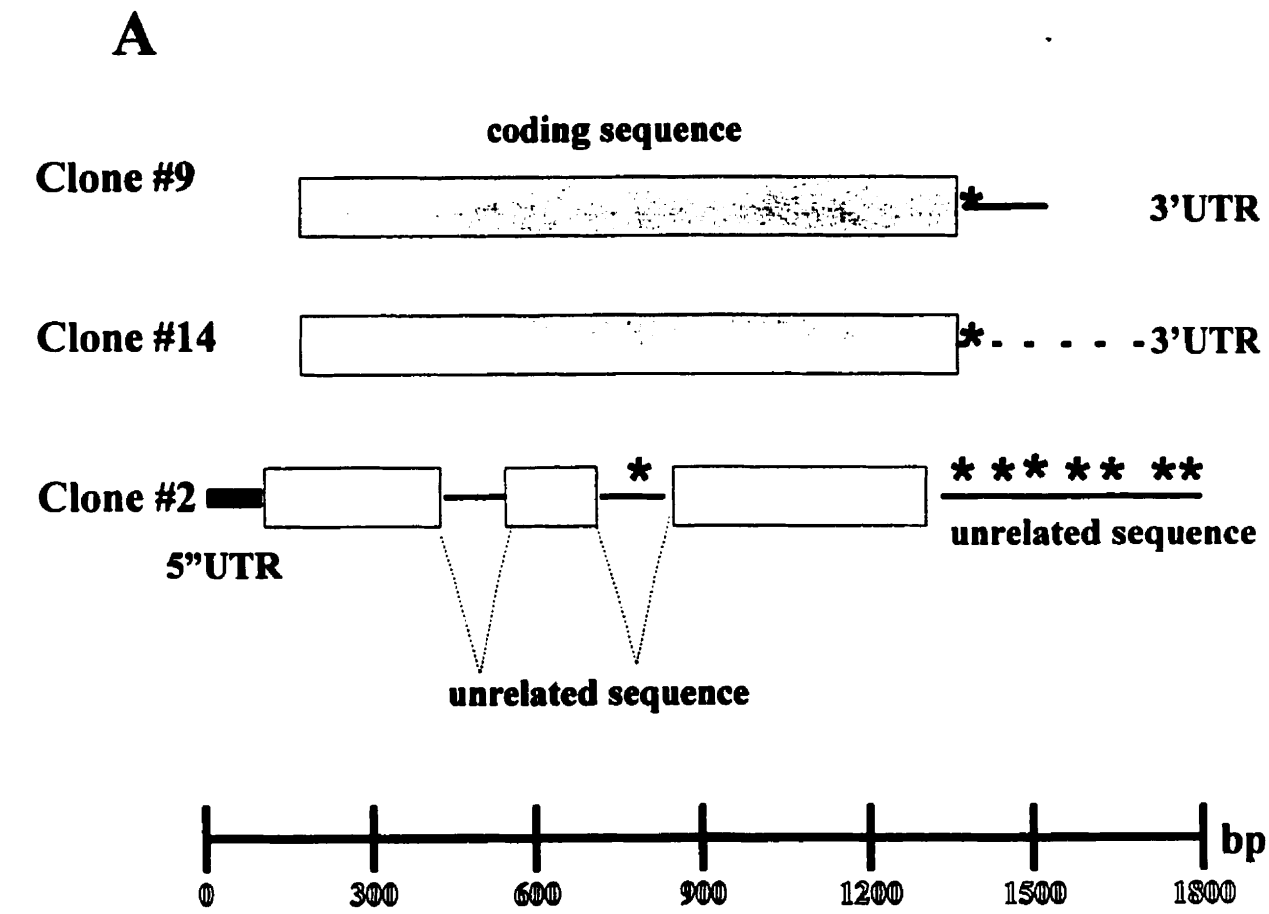


Figure 1: Cloning of the Full-Length Mouse CKI-γ2 cDNA

(A) Schematic diagram showing nucleotide sequences of related CKI-γ2 clones isolated from a mouse brain cDNA library. Clone #9: 1336bp, Clone#14: 1480bp, Clone#2: 1700bp. **(B)** Schematic diagram showing nucleotide sequence for full-length CKI-γ2 #9 and CKI-γ2 #14 cDNAs. Shaded boxes represent CKI-γ2 coding sequences. Asterisks(*) represent stop codons. The thin solid line represents the 3'-untranslated sequence of clone #9 and the thin dashed line represents the 3'-untranslated sequence of clone #14. The thick solid line represents the 5'-untranslated sequence from clone #2.

coding sequence of clone#2 was interrupted by several internal inserts containing stop codons (Fig. 1A). Clone#2 is likely a chimeric cDNA, resulting from cloning artifacts probably introduced during the preparation of the library. A composite sequence was built with the 5'-untranslated sequence and partial coding sequences from clone#2 in conjunction with the coding sequence and the 3'-untranslated sequence from clone#9 (designated CKI-γ2#9 in Fig.1B). A similar composite sequence was constructed from clone#2 and clone#14 (designated CKI-γ2#14 in Fig.1B).

3.1.2 Characterization of Anti-CKI-γ2 Antibodies

It has already been reported (96), that a 75kDa serine/threonine protein kinase coimmunoprecipitated with Nck, presents Casein Kinase I-like enzymatic properties and was recognized by a rabbit polyclonal antibody raised against the C-terminus of CKI-γ2 (1602). Although the results support a constitutive association between Nck and a p75 CKI-γ2, it remains that the published cDNA sequence of CKI-γ2 encodes for a protein of predicted molecular mass of 45.5kDa (74). To investigate the size discrepancy of this immunoreactive p75 CKI-γ2 associated with Nck, a second rabbit polyclonal antibody (1986) was prepared against the full-length CKI-γ2 (residues 1-414) to analyze the specificity of the previous CKI-γ2 antibody (1602), raised against only the C-terminus of CKI-γ2 (residues 192-414). Each anti-CKI-γ2 antibody was verified by subjecting HTC-IR cell lysates to immunoprecipitation and immunoblotting with either 1602 (Fig.2A) or 1986 (Fig.2B) CKI-γ2 antibodies. As can be seen in Figure 2A and 2B, both CKI-γ2 antibodies, 1602 or 1986, but not the normal serum, specifically recognize a protein of approximately 75kDa from HTC-IR cell lysates. In addition, recognition of the 75kDa protein by either CKI-γ2 antibody is prevented by an excess of antigen (data not shown) and therefore confirms that the CKI-γ2 antibodies (1602 and 1986), are specific for recognition of a 75kDa CKI-γ2 in HTC-IR cells.

Figure 2: Characterization of Anti-CKI-γ2 Antibodies

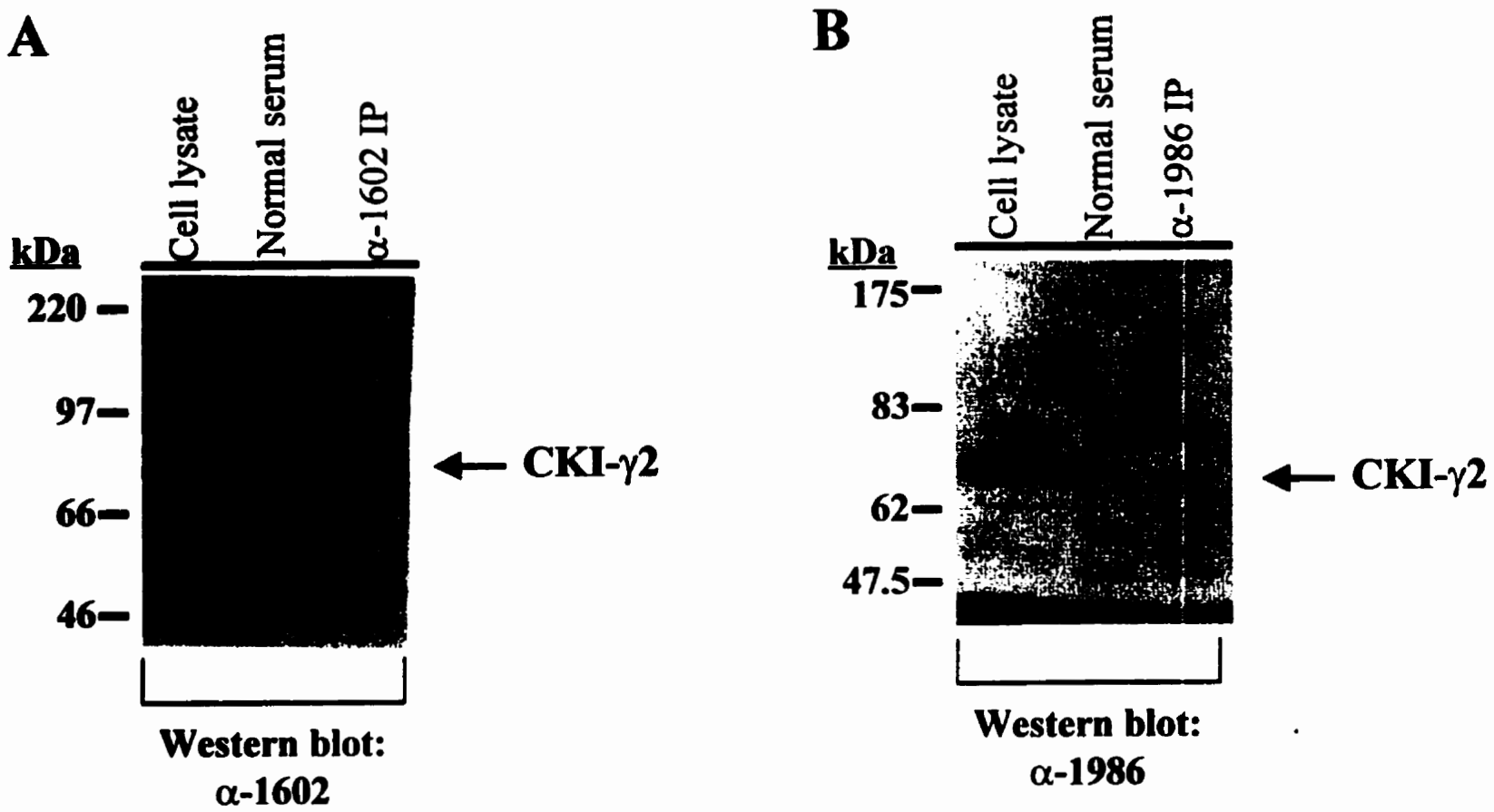


Figure 2: Characterization of Anti-CKI-γ2 Antibodies

Clarified HTC-IR cell lysates prepared in homogenization buffer **(A)** or 1% Triton lysis buffer **(B)** were immunoprecipitated with normal serum, anti-CKI-γ2 serum 1602 **(A)**, or anti-CKI-γ2 serum 1986 **(B)**. Immunoprecipitated proteins and proteins from total cell lysate (20μg) were resolved by SDS-PAGE (7.5% acrylamide gel) and transferred to nitrocellulose. Immunoreactive CKI-γ2 was revealed by immunoblotting with anti-CKI-γ2 antibody 1602 **(A)** or 1986 **(B)**, protein A-HRP and ECL.

3.1.3 Reciprocal Coimmunoprecipitation of CKI-γ2 and Nck

It has been shown that the immunoreactive p75 CKI-γ2 coimmunoprecipitated with Nck from HTC-IR cells (96). However, in this study, the reciprocal coimmunoprecipitation (Nck coprecipitated with the p75 CKI-γ2) was not performed. To fulfill this gap, the experiment was repeated where clarified HTC-IR cell lysates were immunoprecipitated with anti-Nck antibody (UBI#06-288) or anti-CKI-γ2 antibody (1602) and the appropriate immunoblotting was performed. P75 CKI-γ2 was detected in Nck immunoprecipitates by immunoblotting with anti-CKI-γ2 antibody (1602) (Fig.3A) and following immunoblotting with anti-Nck antibody, Nck was detected in CKI-γ2 immunoprecipitates (Fig. 3B). These results clearly demonstrate that the interaction between Nck and p75 CKI-γ2 exists *in vivo*.

3.1.4 Ubiquitous Expression of CKI-γ2 and Nck

To further characterize the immunoreactive p75 CKI-γ2, tissue distribution of CKI-γ2 mRNA was determined by Northern analysis on total RNA from different mouse tissues (adipocyte, brain, heart, kidney, liver, lung, muscle, and testis). Since all mammalian CKI isoforms have highly conserved N-terminal kinase domains (>50% amino acid identity) and the three γ-isoforms of CKI share considerable amino acid identity (69-78%) over their entire length (74), the nucleotide sequences of CKI-γ1, -γ2, and -γ3 were carefully analyzed to design a cDNA probe more specific for CKI-γ2. It was determined that the XmnI/EcoRI fragment consisting of CKI-γ2 C-terminal coding sequence (nucleotides 927-1242) could be used as a cDNA probe for the γ2-isoform since the sequence of the three CKIγ enzymes varies in this area. Using this CKI-γ2 cDNA probe, only a single transcript of 2.4kb, the published size of CKI-γ2 mRNA (74), was detected in all mouse tissues tested (Fig.4A) and therefore suggests that the probe was specific for CKI-γ2. Similarly, a 2.4kb CKI-γ2 transcript was found in total RNA prepared from mouse embryo fibroblasts and adipocytes (3T3-L1 pre-adipocytes and 3T3-L1 adipocytes), rat fibroblasts (Rat-2), and rat transformed hepatocytes

Figure 3: Reciprocal Coimmunoprecipitation of CKI-γ2 and Nck

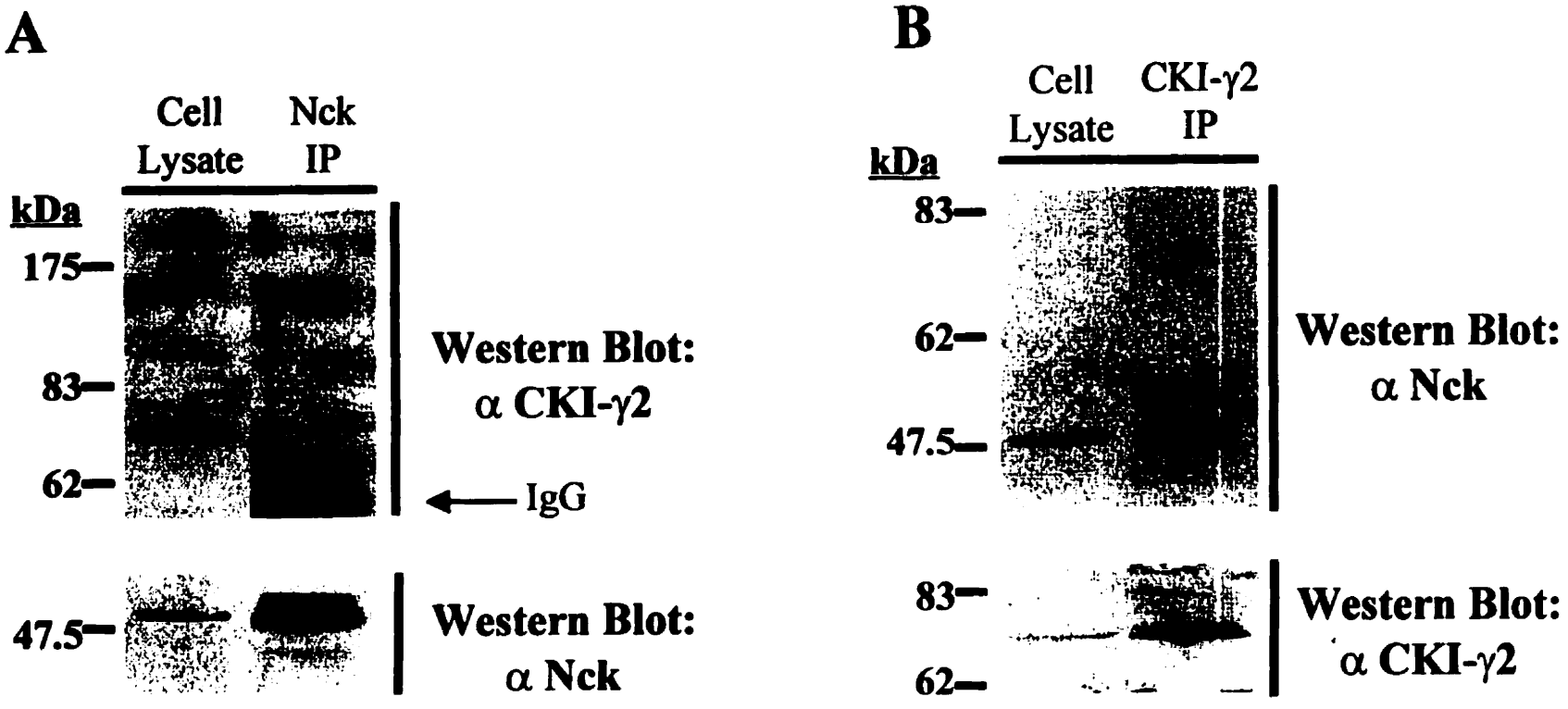


Figure 3: Reciprocal Coimmunoprecipitation of CKI-γ2 and Nck

(A) Clarified HTC-IR cell lysates prepared in 1% Triton lysis buffer were immunoprecipitated with anti-Nck antibody (UBI#06-288). The immunoprecipitated proteins and proteins form total cell lysate were resolved by SDS-PAGE (7.5% acrylamide gel) and transferred to nitrocellulose. CKI-γ2 was revealed by immunoblotting with anti-CKI-γ2 antibody (1602), protein A-HRP, and ECL **(top panel)**. The blot was stripped and Nck was revealed by immunoblotting with anti-Nck antibody (1698), protein A-HRP, and ECL **(bottom panel)**.

(B) In the reciprocal experiment, HTC-IR cell lysates were immunoprecipitated with anti-CKI-γ2 antibody (1602). The immunoprecipitated proteins and proteins from total cell lysate were resolved by SDS-PAGE (7.5% acrylamide gel) and transferred to nitrocellulose. Nck was revealed by immunoblotting with anti-Nck antibody (1698), protein A-HRP, and ECL **(top panel)**. The blot was stripped and CKI-γ2 was revealed by immunoblotting with anti-CKI-γ2 antibody (1602) **(bottom panel)**.

Figure 4: Northern Blot Analysis of CKI-γ2 and Nck

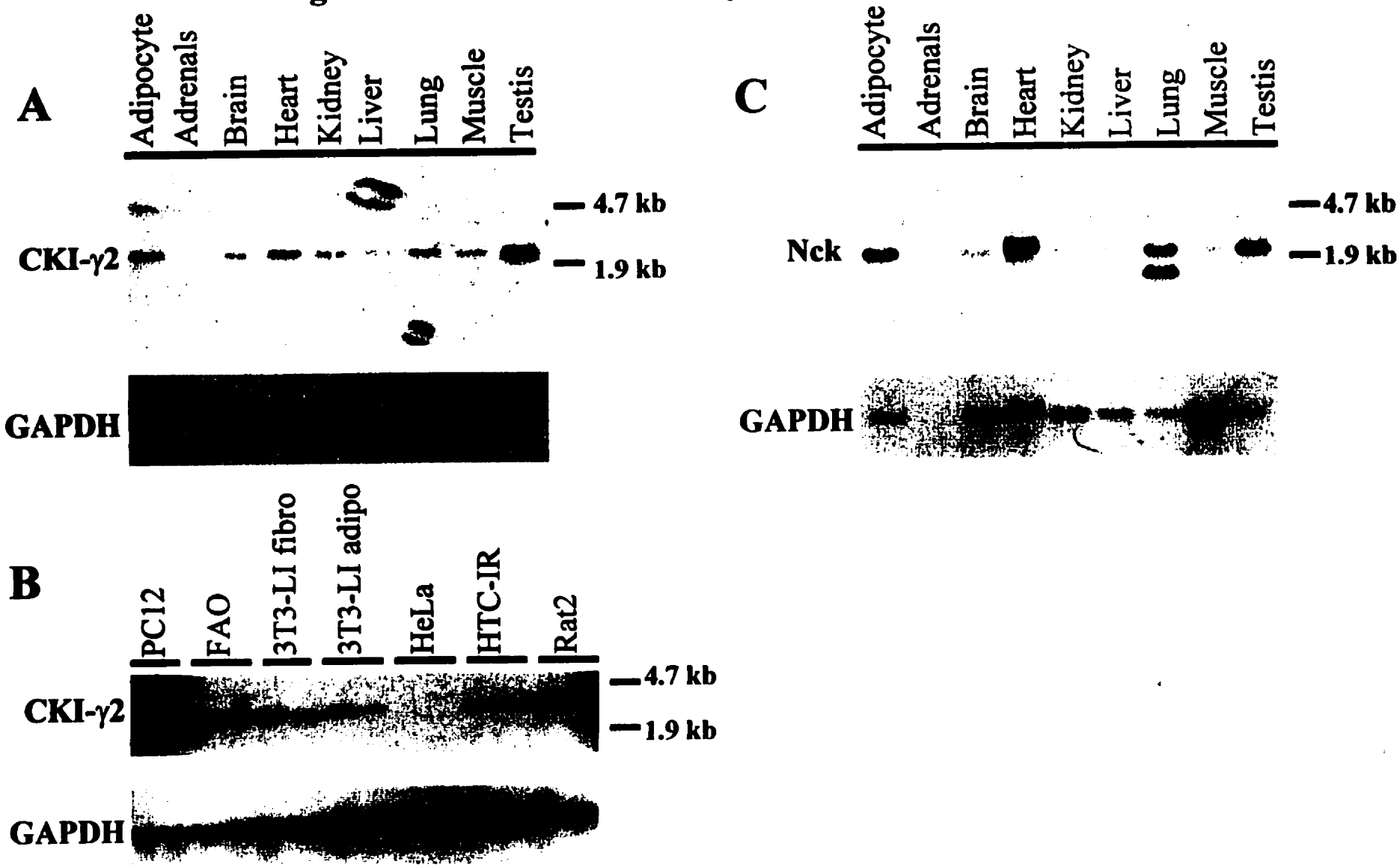


Figure 4: Northern Blot Analysis of CKI-γ2 and Nck

Total RNA was prepared from the indicated mouse tissues and from the indicated mouse, rat, and human cell lines according to procedures described in "Materials and Methods". Equal amounts of total RNA (10μg/lane) were size fractionated by electrophoresis, transferred onto a nylon membane, and hybridized with cDNA probes specific for CKI-γ2 **(top panels of A and B)** or Nck **(top panel of C)**. After detection by autoradiography, the membrane was stripped and reprobed with a GAPDH cDNA probe as control **(bottom panels of A, B, and C)**.

(HTC-IR and FAO) (Fig 4B). No CKI-γ2 transcript was detected in the rat adrenal pheochromocytoma (PC12) and in the human cervix epitheloid carcinoma (HeLa) cell lines. In these cell lines, the level of CKI-γ2 mRNA transcript may be below the level of detection, if not completely absent, or perhaps the mouse CKI-γ2 cDNA probe does not recognize the human CKI-γ2 mRNA transcript.

To examine the expression of Nck mRNA in the different mouse tissues, Northern analysis was performed with a human Nck cDNA probe encompassing its three SH3 domains. A Nck transcript of 2.4kb was ubiquitously expressed in all mouse tissues tested (Fig. 4C) as previously published (98). The faster migrating band in the lung may be the result of alternative splicing of a mRNA encoding a yet unidentified protein that is highly homologous to Nck. In accordance with the findings that Nck and the p75 CKI-γ2 coimmunoprecipitate in rat transformed hepatocytes overexpressing the insulin receptor (HTC-IR cells), it is of interest to find that CKI-γ2 and Nck mRNA are both ubiquitously expressed and therefore found in the same tissues. In addition, mouse testis, lung, and adipocyte tissues, which express the highest levels of CKI-γ2 mRNA, also express the highest levels of Nck mRNA.

Tissue distribution of the immunoreactive p75 CKI-γ2 protein in a variety of mouse tissues was examined by immunoblotting with the anti-CKI-γ2 antibody (1986) (Fig. 5A). Results of Western Blot analysis show that CKI-γ2 is widely expressed only as a 75kDa protein, and with high levels of expression in the kidney, liver, heart, and testis tissues. With longer exposure, p75 CKI-γ2 is also detected in the lung (data not shown). However, a CKI-γ2 of 45.5kDa, the predicted size of CKI-γ2, was not detected in any of the mouse tissues tested. Tissue distribution of Nck protein was also examined in the same mouse tissues by using the anti-Nck antibody (1698) (Fig. 5B). Nck is a 47kDa protein ubiquitously expressed in all mouse tissues tested, and with high levels of expression in testis, brain, lung, and adipocyte. Interestingly, both Nck and the

Figure 5: Western Blot Analysis of CKI-γ2 and Nck

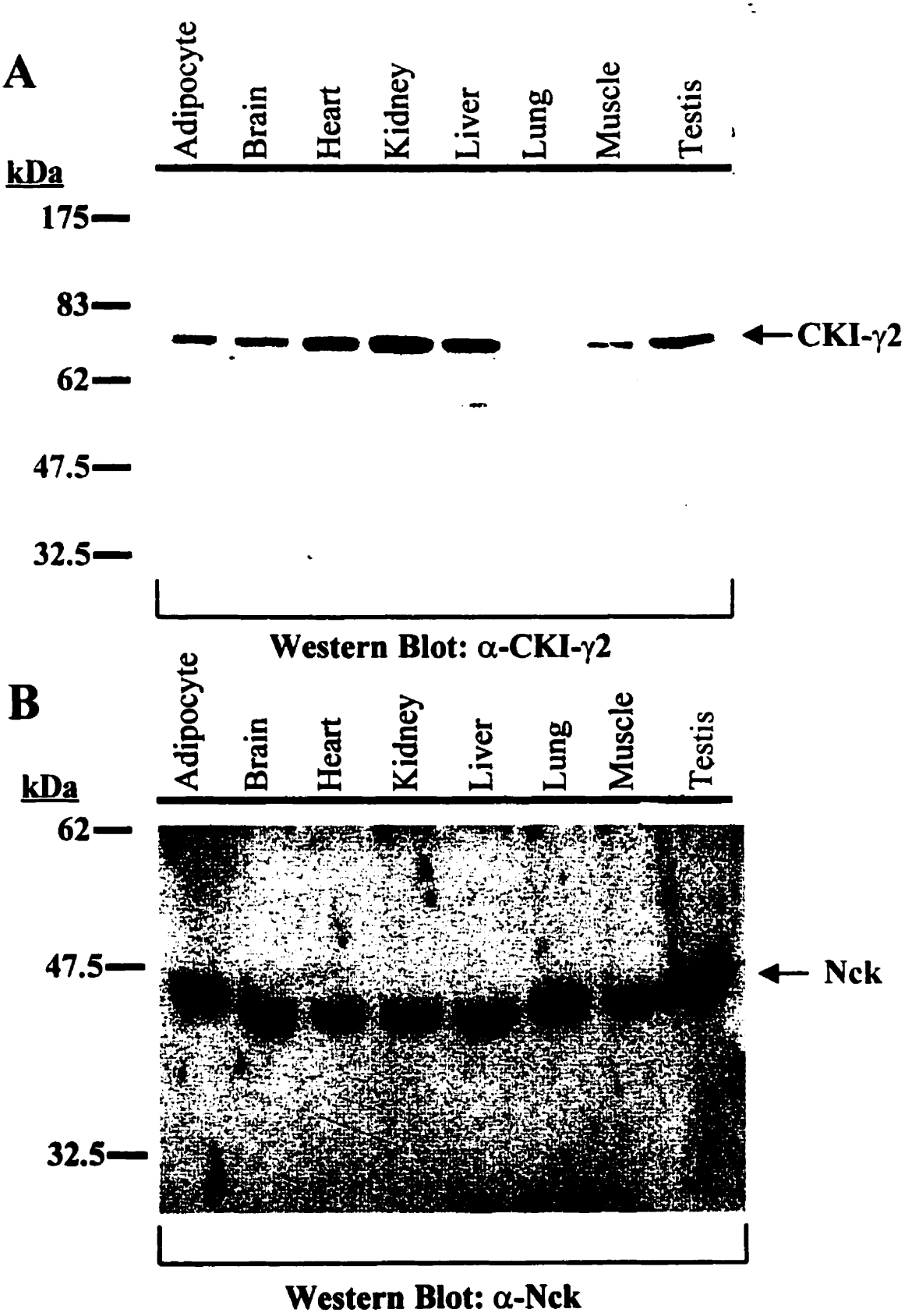


Figure 5: Western Blot Analysis of CKI-γ2 and Nck

Homogenates of the indicated mouse tissues were prepared according to procedures described in "Materials and Methods". Equal amounts of protein (100μg per tissue) were resolved by SDS-PAGE, and transferred to nitrocellulose. CKI-γ2 or Nck was detected by immunoblotting with anti-CKI-γ2 antibody (1986) **(A)** or anti-Nck antibody (1698) **(B)**, respectively, followed by ^{125}I-GAR and autoradiography.

p75 CKI-γ2 are highly expressed in testis tissues. The ubiquitous immunological detection of only one band of 75kDa for CKI-γ2 in all murine tissues tested, strongly supports its existence as a 75kDa protein.

3.1.5 Expression and Kinase Activity of Recombinant CKI-γ2

An initial step in the characterization of the immunoreactive p75 CKI-γ2 activity was to produce the recombinant enzyme by cloning CKI-γ2 coding sequence into pGEX4T2. A culture of *E. Coli* strain XLI-Blue MR transformed with the CKI-γ2 pGEX4T2 plasmid was induced and the cells lysed as described under "Materials and Methods". GST-CKIγ2 was immobilized on glutathione-agarose beads (designated, GST-CKIγ2 on beads) according to the manufacturer's instructions (Pharmacia) and determined to be a 75kDa protein in Coomassie Blue-stained gels (Fig. 6A). A sample of BSA (67kDa) migrates just below the 75kDa GST-CKIγ2 in SDS-PAGE. GST-CKIγ2 is expressed as an active enzyme as evidenced by the autophosphorylation of the recombinant protein in the presence of ATP and Mg^{2+} (Fig. 6B). GST-CKIγ2 activity was also confirmed by phosphorylation of the exogenous substrate, α-casein, a preferred substrate of CKI family members (2, 3) (Fig. 6C). In addition, GST-CKIγ2 was eluted from glutathione-agarose beads (designated, eluted GST-CKIγ2) according to the manufacturer's recommendations (Pharmacia) and its activity was confirmed by an *in vitro* kinase assay of the recombinant enzyme alone or in the presence of α-casein (Fig. 6D). The results demonstrate that the recombinant GST-CKIγ2 behaves as an active kinase that can be used for studying the enzyme.

3.1.6 Comparison of Recombinant CKI-γ2, Endogenous CKI-γ2, and the CKI-like Protein Associated with Nck Revealed Similar Enzymatic Characteristics

GST-CKIγ2 expressed in bacteria, has an apparent molecular mass of ≈75kDa, of which, 27kDa accounts for the GST portion and the remainder, ≈ 48kDa, is in agreement with the estimated size of CKI-γ2 (74). However, the immunoreactive

Figure 6: Expression and Kinase Activity of Recombinant CKI-γ2

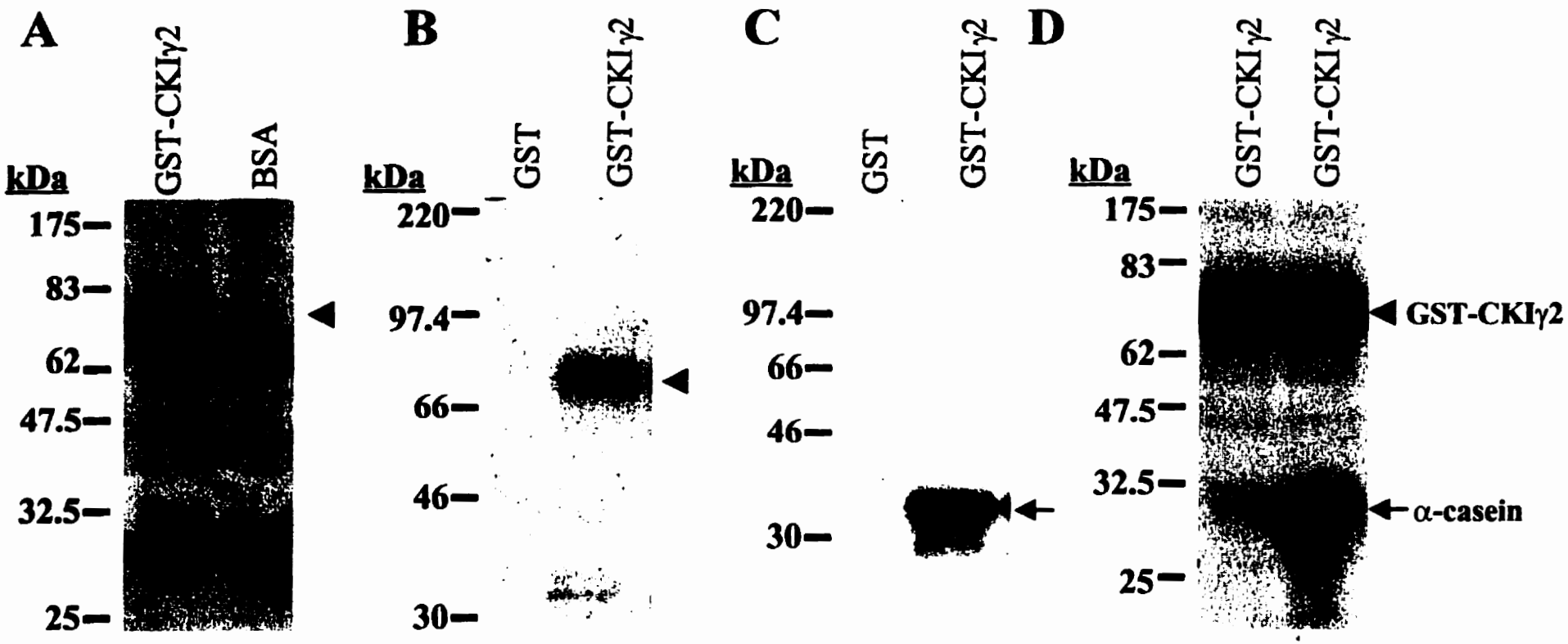


Figure 6: Expression and Kinase Activity of Recombinant CKI-γ2

CKI-γ2 cDNA was subcloned into a pGEX4T2 vector and the protein expressed in *E. Coli*. The fusion protein, GST-CKIγ2, was prepared and immobilized on glutathione-agarose beads (designated, GST-CKIγ2 on beads) according to the manufacturer's recommendations (Pharmacia). **(A)** GST-CKIγ2 on beads and Bovine Serum Albumin (BSA) were resolved by SDS-PAGE (7.5% acrylamide gel) and stained with Coomassie blue. **(B)** GST or GST-CKIγ2 on beads were subjected to *in vitro* kinase assays as indicated in "Materials and Methods". The samples were subjected to SDS-PAGE (12% acrylamide gel) and autoradiography. **(C)** Similarly, GST or GST-CKIγ2 on beads were subjected to *in vitro* kinase assays but in the presence of an exogenous substrate, α-casein (5μg). **(D)** GST-CKIγ2 was eluted from beads (designated, eluted GST-CKIγ2) according to the manufacturer's recommendations (Pharmacia) and subjected to *in vitro* kinase assays alone or in the presence of α-casein (5μg). The arrow head indicates GST-CKIγ2 and the arrow represents α-casein.

CKI-γ2 coimmunoprecipitated with Nck exhibits the enzymatic characteristics of a CKI-like kinase activity (96) and is 75kDa. To confirm that the CKI-like kinase activity coimmunoprecipitated with Nck is indeed CKI-γ2, its enzymatic characteristics were compared to those of recombinant CKI-γ2 and endogenous immunoprecipitated CKI-γ2.

The first enzymatic characteristic accessed was the ionic requirements of each source of CKI-γ2 protein (Fig. 7A). *In vitro* kinase assays were performed on GST-CKIγ2, CKI-γ2 immunoprecipitates, or Nck immunoprecipitates in the presence of increasing concentrations of magnesium chloride ($MgCl_2$) or manganese chloride ($MnCl_2$). All three sources of CKI-γ2 protein showed optimal kinase activity toward α-casein with 5-10mM of $MgCl_2$. Although some kinase activity is observed with high concentrations of $MnCl_2$, a mixture of magnesium (10mM) and manganese (5mM) inhibits the kinase activity of all three sources of CKI-γ2 protein (data not shown). The results agree with previous reports that CKI-γ2 is a magnesium-dependent kinase (74). Secondly, the substrate specificity of each source of kinase activity was tested on a variety of exogenous substrates (Fig. 7B). In all three sources of CKI-γ2 protein, α-casein and phosvitin, two preferred substrates of members of the CKI family (2, 3), as well as myelin basic protein (MBP) were phosphorylated *in vitro*. β-Casein and PolyGluTyr, a substrate of tyrosine kinases, were not efficient substrates. Lastly, the dose-dependent effect of the specific CKI inhibitor, N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide (CKI-7) (15), is shown in figure 7C. CKI-7 inhibited the *in vitro* phosphorylation of α-casein by recombinant CKI-γ2, endogenous CKI-γ2, and the CKI-like protein associated with Nck. GST-CKIγ2 activity was inhibited by CKI-7 with an IC_{50} of 60.3μM, and is identical to the published IC_{50} value for CKI-7 inhibition of another recombinant CKI-γ2 (74). The IC_{50} values for CKI-7 inhibition of kinase activity associated with CKI-γ2 immunoprecipitates and Nck immunoprecipitates, 21μM and 20μM respectively, are in the range of concentrations for the inhibitory effect of CKI-7 on purified

Figure 7: Comparison of Recombinant CKI-γ2, Endogenous CKI-γ2, and the CKI-like Protein Associated with Nck Revealed Similar Enzymatic Characteristics

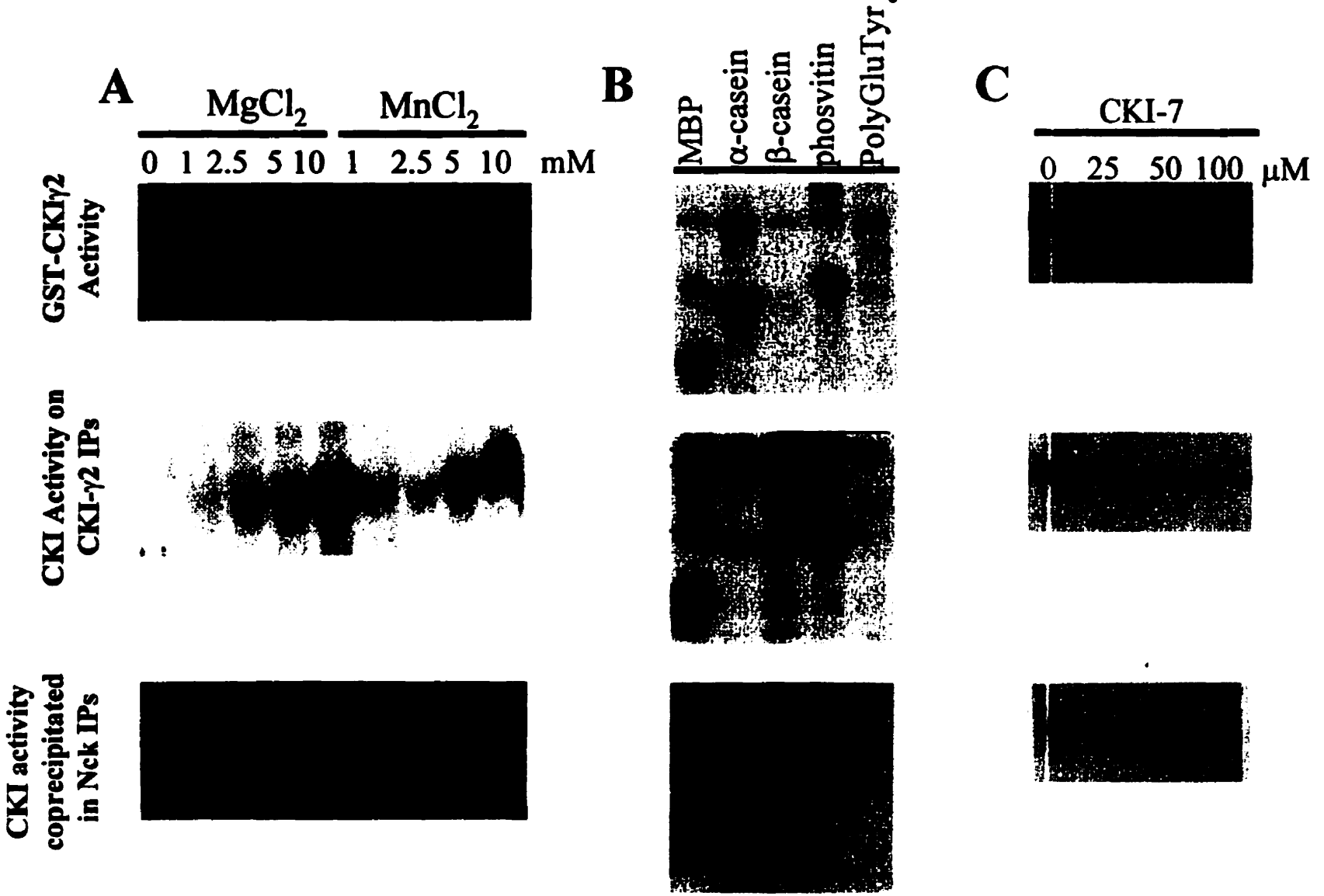


Figure 7: Comparison of Recombinant CKI-γ2, Endogenous CKI-γ2, and the CKI-like Protein Associated with Nck Revealed Similar Enzymatic Characteristics

The enzymatic characteristics of recombinant CKI-γ2 (GST-CKI-γ2 activity), endogenous CKI-γ2 (CKI-γ2 activity on CKI-γ2 IPs), and the CKI-like protein associated with endogenous Nck (CKI activity coprecipitated in Nck IPs) were analyzed by *in vitro* kinase assays in the presence of α-casein (5μg) as described in "Materials and Methods". **(A)** Ion dependence was determined by the addition of 0, 1, 2.5, 5, or 10mM $MgCl_2$ or $MnCl_2$ to the assay containing ion-free Kinase Buffer. **(B)** Substrate specificity was determined by the addition of 5μg of myelin basic protein (MBP), 15μg of α-casein, 15μg of β-casein, 15μg of phosvitin, or 15μg of PolyGluTyr. **(C)** The effect of CKI-7 was determined by the addition of 0, 25, 50, or 100μM of CKI-7. All kinase assays were analyzed by SDS-PAGE and autoradiography.

(15) or recombinant CKI activity (74, 76).

3.1.7 Murine CKI-γ2 is not a Dual-Specific Kinase

It has been reported that some of the yeast homologs of CKI possess dual-specific kinase activity and autophosphorylate on serine, threonine, and tyrosine residues (69). CKI-γ2 was therefore accessed for dual-specific kinase activity, by analyzing the *in vitro* autophosphorylation of increasing amounts of eluted GST-CKIγ2 (negative data not shown). The reasoning is that if CKI-γ2 is a dual-specific kinase capable of transferring phosphate to serine, threonine, and tyrosine residues, then it should be autophosphorylated on tyrosine residues and detected by immunoblotting with anti-phosphotyrosine antibody. The results show that GST-CKIγ2 is not recognized by anti-phosphotyrosine antibody (RC20) and therefore suggest that mammalian CKI-γ2 is not a dual-specific kinase. The results are consistent with previous findings that PolyGluTyr was not phosphorylated by any of the three sources of CKI-γ2 (see Fig.7B).

3.1.8 Phosphatase Treatment of Immunoprecipitated CKI-γ2 from HTC-IR Cells

Since a 75kDa CKI-γ2 has never been reported, an explanation for the size discrepancy of CKI-γ2 was investigated. One possibility is that the p75 CKI-γ2 is the result of a post-translational modification of the kinase in cells. One common post-translational modification of signaling molecules is phosphorylation. To determine if p75 CKI-γ2 is a hyperphosphorylated form of the kinase, CKI-γ2 immunoprecipitates from HTC-IR cells were subjected to phosphatase treatment to see if a dephosphorylated 45kDa CKI-γ2 could be observed (Fig. 8). The two phosphatases utilized were Potato Acid Phosphatase (PAP), a general phosphatase of phosphoserine, phosphothreonine, and phosphotyrosine residues, and Protein Phosphatase 2A (PP2A), a more specific phosphatase of phosphoserine and phosphothreonine residues. Phosphatase-treated or untreated CKI-γ2 immunoprecipitates were immunoblotted with anti-CKI-γ2 antibody and the

Figure 8: Phosphatase Treatment of Immunoprecipitated CKI-γ2 from HTC-IR Cells

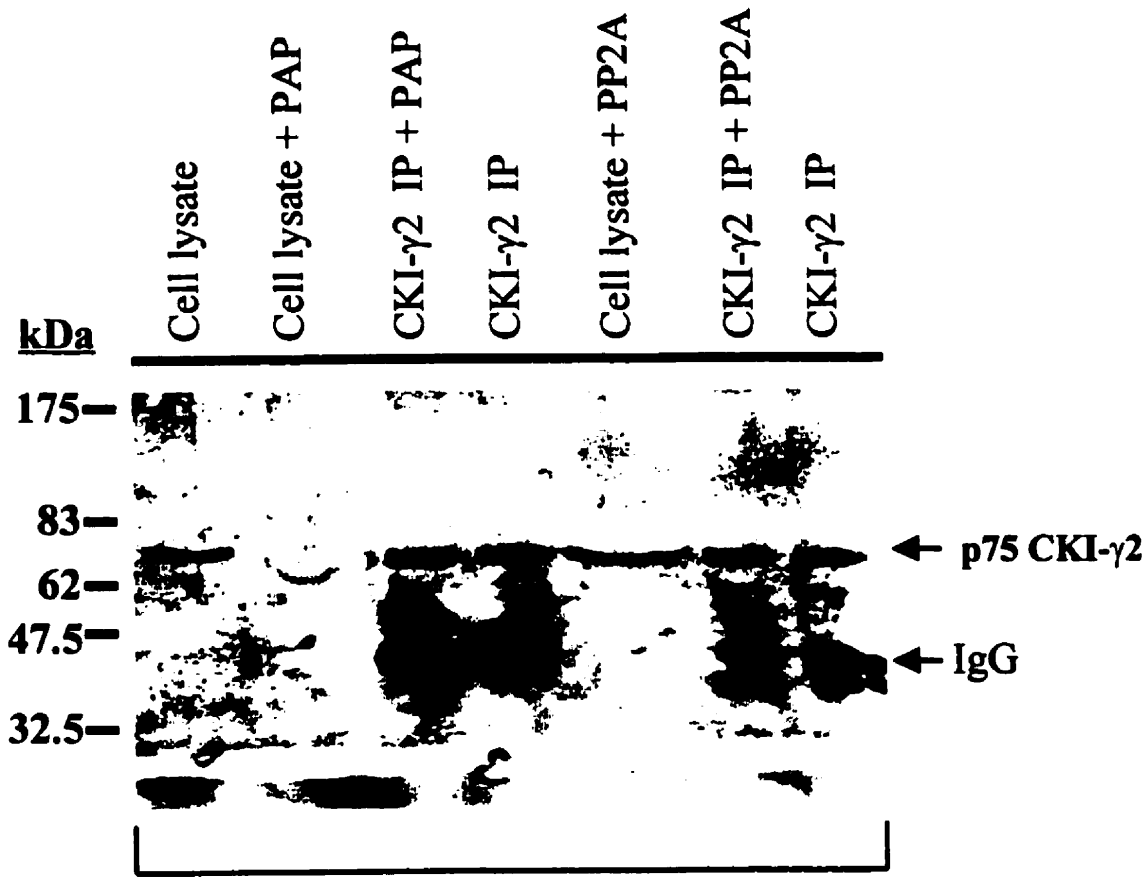


Figure 8: Phosphatase Treatment of Immunoprecipitated CKI-γ2 from HTC-IR Cells

Clarified HTC-IR cell lysates prepared in 1% Triton lysis buffer were immunoprecipitated with anti-CKI-γ2 antibody (1602). Washed CKI-γ2 immunoprecipitates were dephosphorylated with potato acid phosphatase (PAP) (6U/ml) or with protein phosphatase 2A catalytic subunit (PP2A) (20mU/ml) for 30min at 37°C. Phosphatase-treated and untreated CKI-γ2 immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-CKI-γ2 antibody (1602), protein A-HRP, and ECL.

results show that, in general, no decrease in signal associated with the immunoreactive p75 CKI-γ2 nor any lower molecular weight species that could correspond to a dephosphorylated CKI-γ2 was observed. The lower approximately 55kDa band observed in some samples is also observed in control samples that were not treated with any phosphatase, and therefore do not correspond to a dephosphorylated CKI-γ2.

3.1.9 *In Vitro* Transcribed/Translated CKI-γ2 is an Active Kinase of 45kDa Recognized by CKI-γ2 Antibody

To see if a post-translational modification other than phosphorylation of CKI-γ2 can be observed in an *in vitro* system, CKI-γ2 cDNA was transcribed and translated, *in vitro,* in both rabbit reticulocyte and wheat germ extract systems (Fig. 9A). [^{35}S]-methionine labeled CKI-γ2 was detected as an approximately 45kDa translation product in both systems. *In vitro* transcription/translation of the luciferase DNA (62kDa protein) was used as a positive control. The 45kDa *in vitro* transcribed/translated CKI-γ2 product was specifically immunoprecipitated by anti-CKI-γ2 antibody (1986) (Fig. 9B), and is an active kinase capable of autophosphorylation and phosphorylation of α-casein, *in* vitro (Fig. 9C). To determine if a post-translational modification of CKI-γ2 could be a transient phenomena, a time-course of *in vitro* transcribed/translated CKI-γ2 was performed with the rabbit reticulocyte system (Fig. 9D). The results show that as early as 15 minutes after the initiation of the reaction, the 45kDa CKI-γ2 protein is translated and does not undergo any further modification that alters its molecular weight, despite a longer time of reaction. Using *in vitro* rabbit reticulocyte and wheat extract systems, CKI-γ2 is produced as 45.5kDa protein in agreement with its published predicted molecular mass.

3.1.10 CKI-γ2 Overexpressed in 293 and Cos-1β Cells is a 45kDa Protein

The fact that the *in vitro* transcription/translation systems produced only a 45kDa CKI-γ2 may not exclude that in cells, this protein could be post-translationally

Figure 9: *In Vitro* Transcribed/Translated CKI-γ2 is an Active Kinase of 45 kDa Recognized by CKI-γ2 Antibody

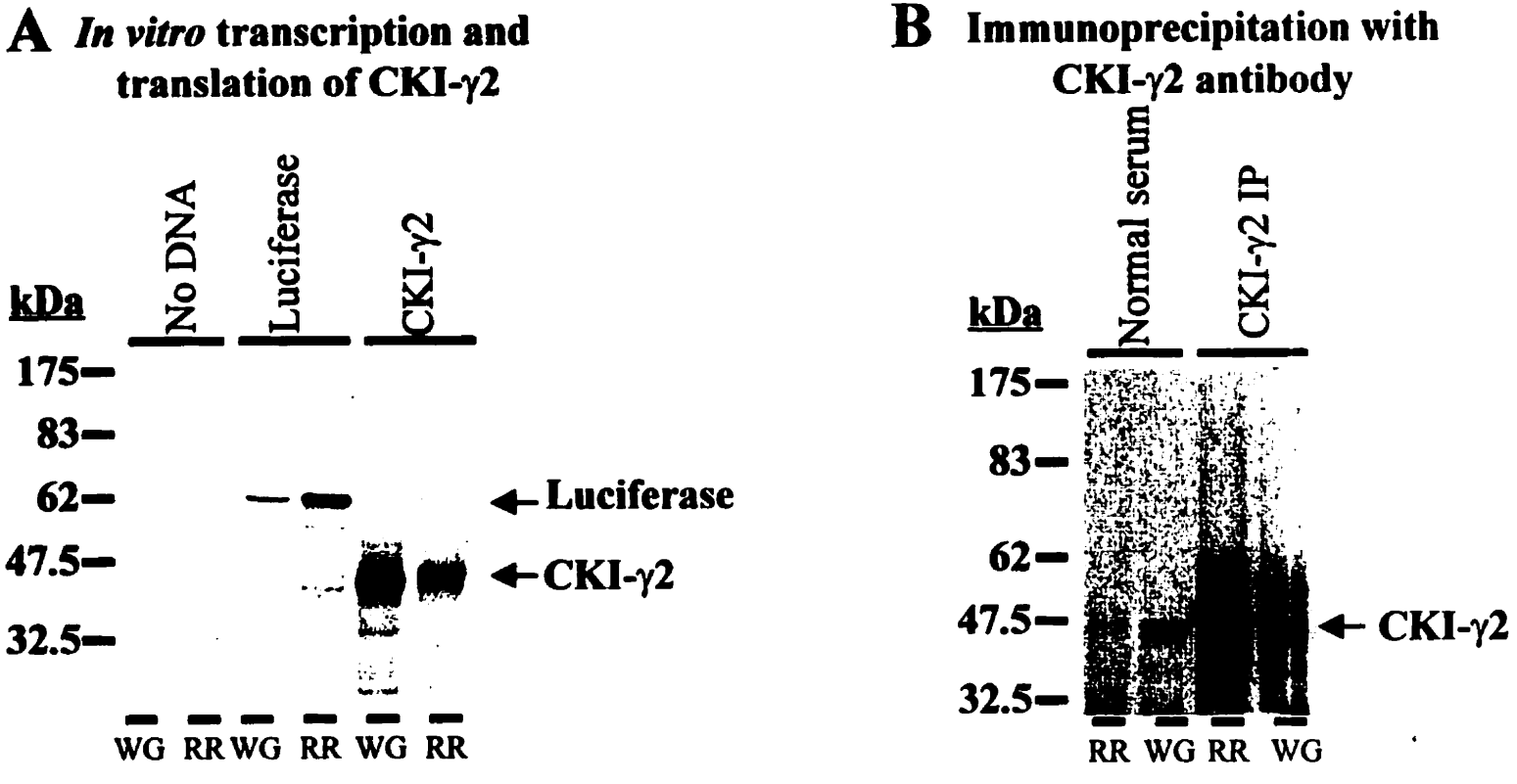


Figure 9: *In Vitro* Transcribed/Translated CKI-γ2 is an Active Kinase of 45 kDa Recognized by CKI-γ2 Antibody

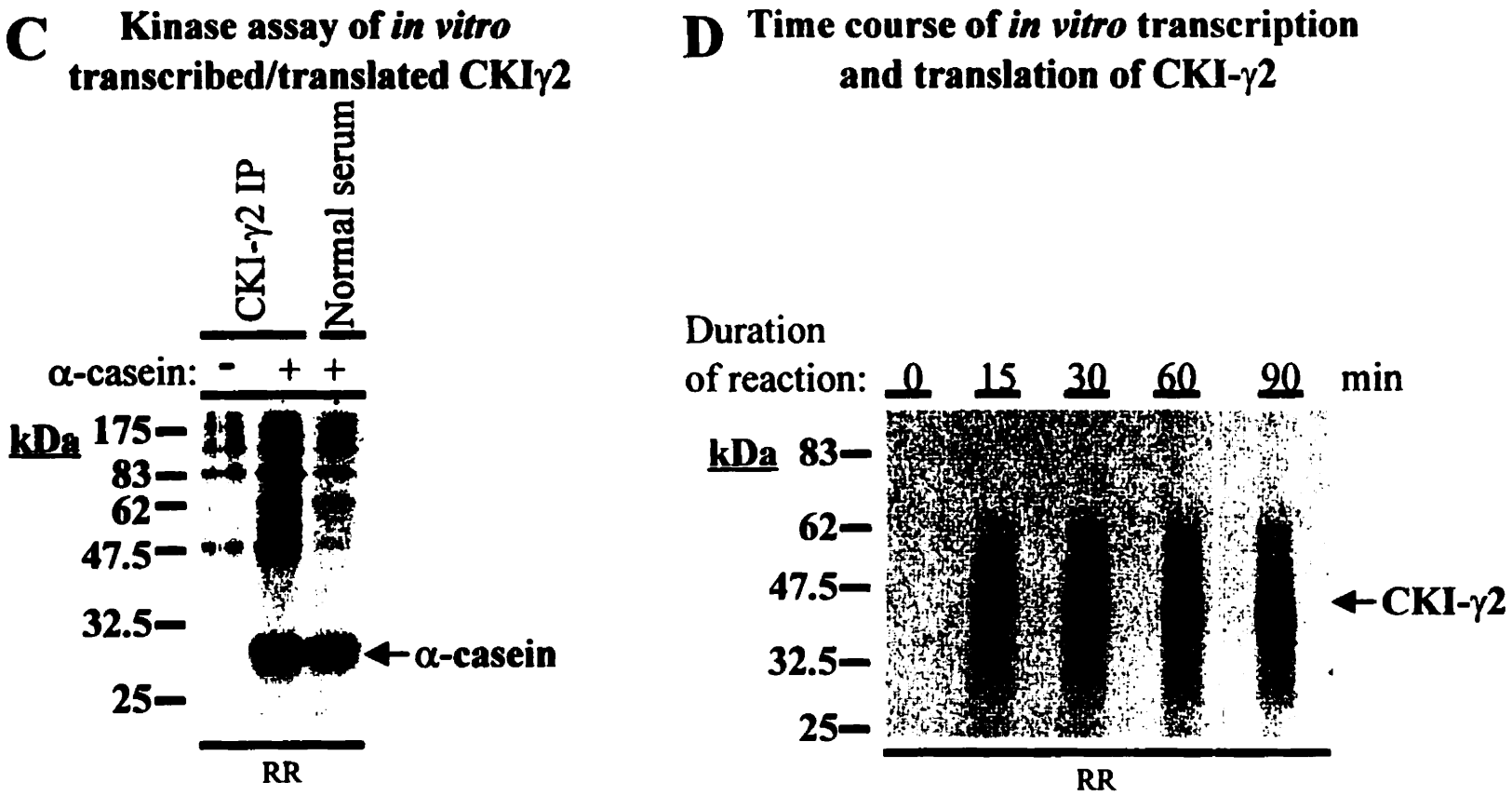


Figure 9: *In Vitro* Transcribed/Translated CKI-γ2 is an Active Kinase of 45kDa Recognized by CKI-γ2 Antibody. **(A)** *In vitro* transcription (from the T7 promoter) and translation (in the presence of [^{35}S]-methionine/cysteine) of 1μg of CKI-γ2 pcDNA3.1+ was carried out using the TNT T7 coupled Rabbit Reticulocyte (RR) and Wheat Germ (WG) extract systems according the manufacturer's instructions (Promega). **(B)** [^{35}S]-labeled *in vitro*-translated CKI-γ2 was immunoprecipitated with normal serum or anti-CKI-γ2 antibody (1986). **(C)** [^{35}S]-labeled *in vitro*-translated CKI-γ2 was immunoprecipitated with anti-CKI-γ2 antibody (1986), and submitted to an *in vitro* kinase assay in the presence or absence of α-casein (5μg). **(D)** *In vitro* transcription and translation of CKI-γ2 was carried out for 15, 30, 60, or 90min, using the TNT T7 coupled Rabbit Reticulocyte (RR) system. For all samples, following resolution by SDS-PAGE, the gels were prepared for fluorography using EN^{3}HANCE, and then exposed for autoradiography.

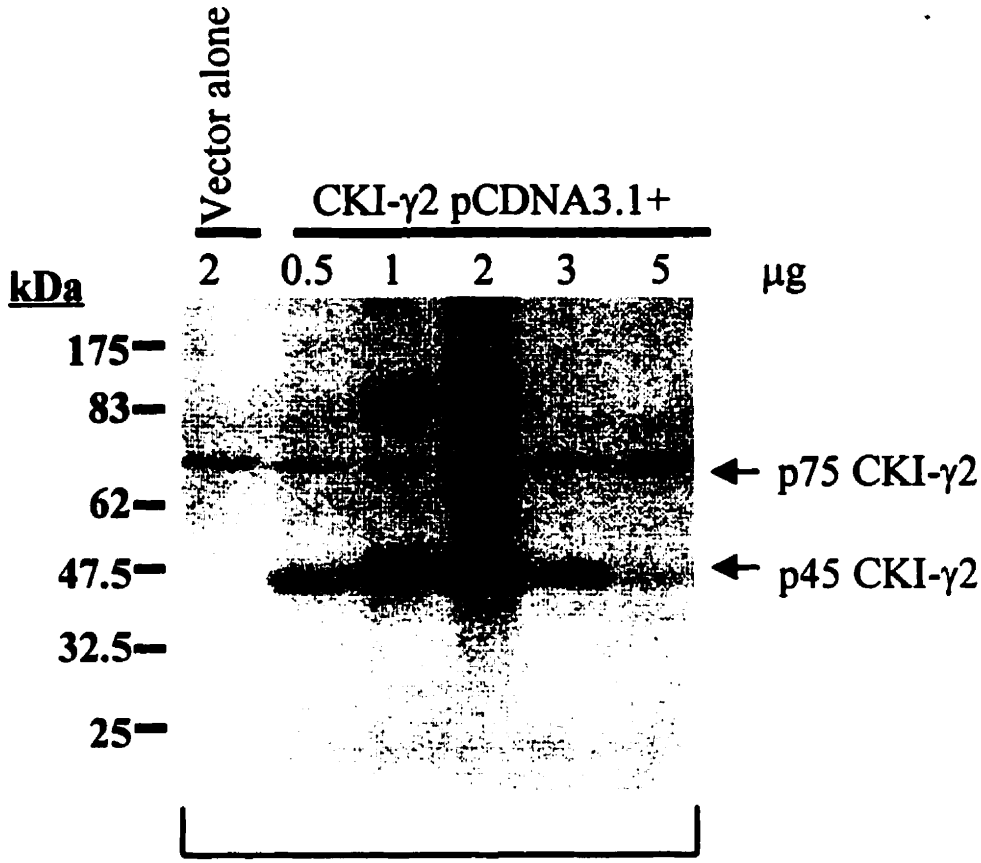
modified leading to a protein of higher molecular weight. To determine if a post-translational modification of CKI-γ2 can be observed in an *in vivo* system, transient transfections of the coding CKI-γ2 cDNA subcloned into pcDNA3.1+ was performed in 293 cells (Fig. 10), and analyzed by immunoblotting with anti-CKI-γ2 antibody (1986). A 45kDa CKI-γ2 was expressed in cells that had been transfected with CKI-γ2 pcDNA 3.1+ as compared to the control cells transfected with the empty vector only. However, an immunoreactive 75kDa CKI-γ2 was detected in all transfected cells including control cells. This p75 CKI-γ2 is the endogenous enzyme that is recognized by anti-CKI-γ2 antibody as observed in previous immunoblot analysis (see Fig. 2, 3, and 5). Similar results were observed in transfected Cos-1β cells (data not shown). As is often observed, a high quantity of DNA is toxic to cells and therefore less p45 CKI-γ2 is observed in Figure 10 with increasing amounts of transfected DNA.

3.2 Objective #2: To determine if Nck is phosphorylated by CKI-γ2.

3.2.1 *In Vitro* Phosphorylation of Nck by CKI-γ2

The interaction of CKI-γ2, a serine/threonine kinase, with the adaptor protein, Nck, in cells led to the examination of whether Nck is, *in vitro*, an effective substrate for phosphorylation by recombinant GST-CKIγ2. Various domains of Nck expressed as GST-fusion proteins were used to evaluate whether Nck could be phosphorylated by CKI-γ2. We have observed that the three SH3 domains of Nck were *in vitro* phosphorylated by CKI-γ2 (Fig. 11A). In contrast, GST and the SH2 domain of Nck were not phosphorylated. In this approach, we have not been able to determine whether the full-length Nck was efficiently phosphorylated because GST-Nck and GST-CKIγ2 comigrate at the same position, and the autophosphorylated GST-CKIγ2 masks any signal associated with the phosphorylation of GST-Nck. To determine which region of the three SH3 domains of Nck is efficiently phosphorylated by CKI-γ2, each individual SH3 domains were submitted to an *in vitro* kinase assay in the presence of GST-CKIγ2

Figure 10: Transient Transfection of CKI-γ2 in 293 Cells



Western Blot: α-CKI-γ2

Figure 10: Transient Transfection of CKI-γ2 in 293 Cells

Lipofectamine-mediated transient transfection of CKI-γ2 pCDNA3.1+ DNA in 293 cells was performed according to the manufacturer's protocol (Life Technologies). Transfections were carried out with 0.5, 1, 2, 3, and 5μg of CKI-γ2 pCDNA3.1+ (CKI-γ2 pCDNA3.1+) and 2μg pcDNA3.1+ alone (vector alone). The cells were harvested 48-72 h after the start of transfection, resolved by SDS-PAGE, and transferred to nitrocellulose. Transient transfections of CKI-γ2 pcDNA 3.1+ were analyzed by immunoblotting with anti-CKI-γ2 antibody (1986), ^{125}I-GAR, and autoradiography.

Figure 11: *In Vitro* Phosphorylation of Nck by CKI-γ2

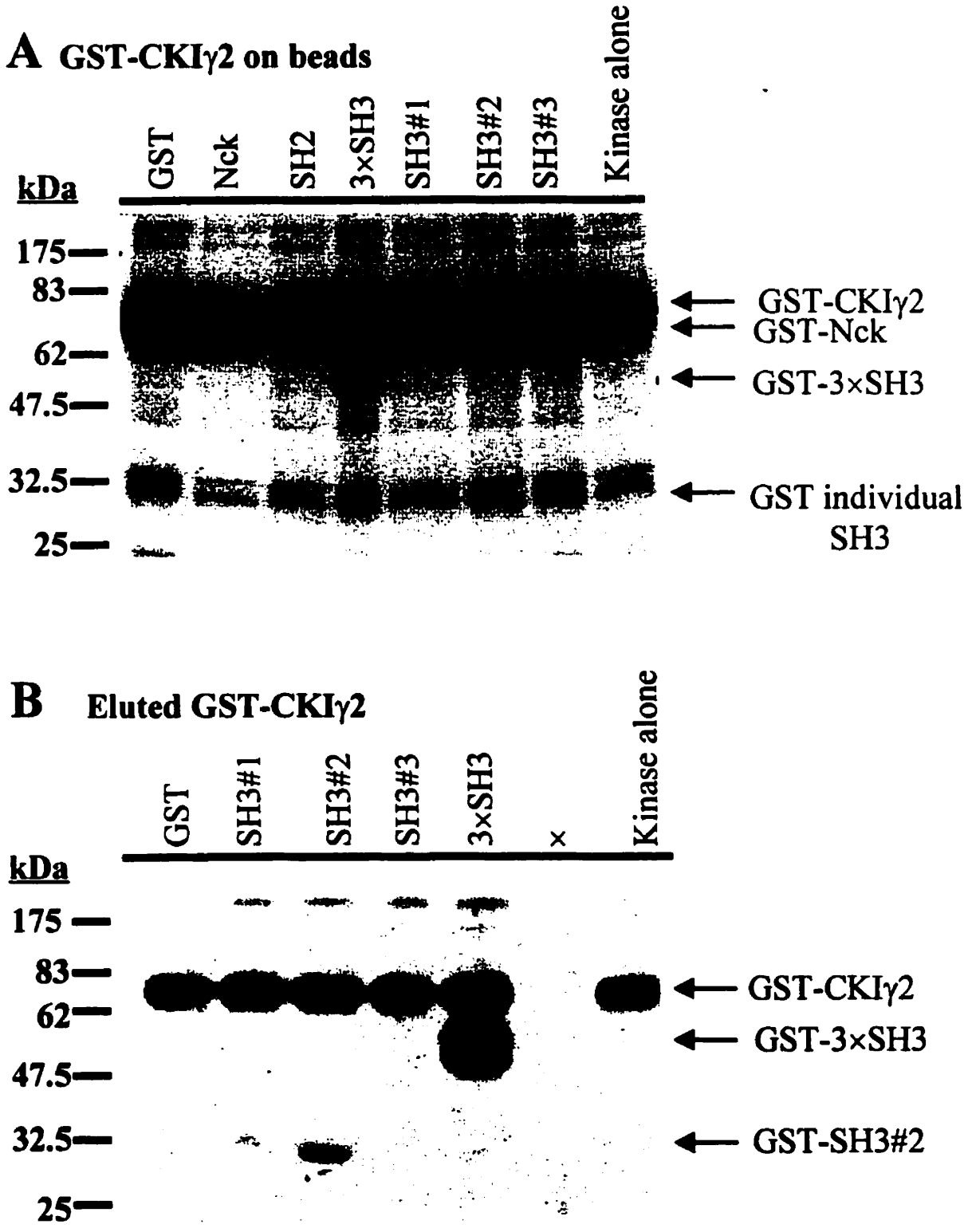


Figure 11: *In Vitro* Phosphorylation of Nck by CKI-γ2

C **GST-Nck 3×SH3 Phosphopeptide Map Analysis**

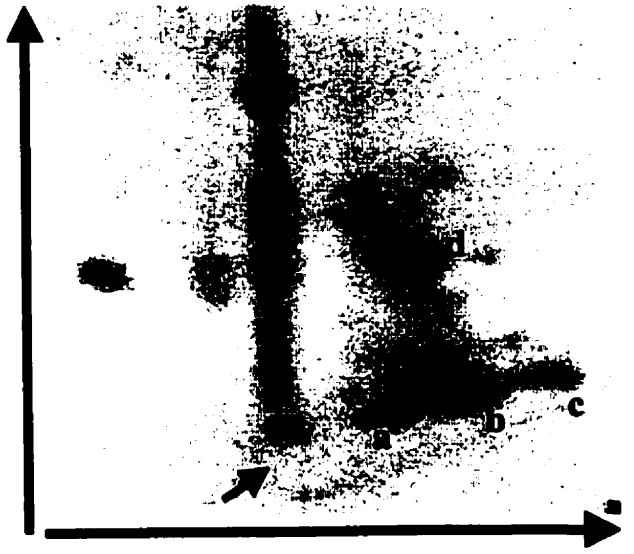


Figure 11: *In Vitro* Phosphorylation of Nck by CKI-γ2

(A) *In vitro* kinase assays were performed with 1μg of GST-CKIγ2 on beads in the presence of various exogenous substrates. The exogenous substrates include 2μg of GST-SH3#1, GST-SH3#2, GST-SH3#3, GST-3×SH3, GST-SH2, GST-Nck, or GST alone. The samples were subjected to SDS-PAGE, transferred to nitrocellulose, and exposed for autoradiography. **(B)** *In vitro* kinase assays were performed with 1μg of eluted GST-CKIγ2 as indicated for A. **(C)** Phosphorylated GST-3×SH3 was subjected to proteolytic phosphopeptide mapping by two-dimensional separation on thin-layer cellulose (TLC) plates as described in "Materials an Methods".

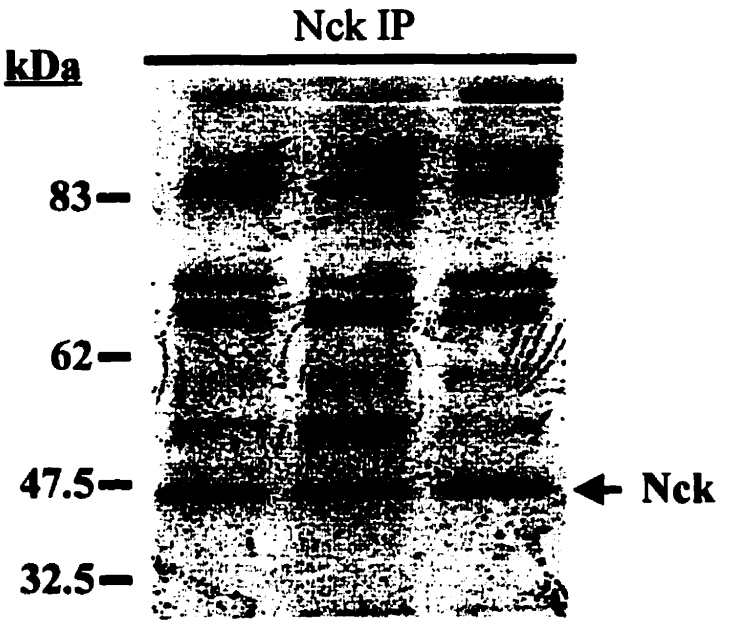
(Fig. 11B). Among the three individual SH3 domains of Nck, only the second SH3 was found phosphorylated, although to a lesser degree than the three SH3 domains together. Similar results were obtained whether a GST-CKIγ2 was immobilized on glutathione-agarose beads (designated GST-CKIγ2 on beads, Fig. 11A) or eluted (designated eluted GST-CKIγ2, Fig. 11B). *In vitro* phosphorylated GST-3×SH3 was localized on nitrocellulose membrane by autoradiography and the corresponding band excised and subjected to tryptic phosphopeptide mapping (Fig. 11C). The *in vitro* phosphorylation of Nck's SH3 domains by CKI-γ2, shared some of the phosphopeptides (b, d, and e in Fig. 11C) obtained from *in vivo* [^{32}P]-labeled Nck isolated from murine NIH 3T3 cells (3, 6, and 7 in ref.109).

3.2.2 *In Vivo* Phosphorylation of Nck

To investigate if Nck is phosphorylated by CKI-γ2 in cells, HTC-IR cells were metabolically labeled for 4h with [^{32}P]-orthophosphate (Fig. 12A). Nck immunoprecipitates from [^{32}P]-labeled cells are shown in figure 12A. The results show that Nck is a highly phosphorylated protein in metabolically labeled HTC-IR cells. [^{32}P]-labeled Nck was localized on nitrocellulose by autoradiography and subjected to tryptic phosphopeptide mapping (Fig. 12B). The phosphopeptide map of *in vivo* [^{32}P]-labeled Nck from HTC-IR cells is similar to that of Nck from NIH 3T3 cells (109) and is therefore numbered according to the published map. Furthermore, phosphopeptides 3 and 7 from the *in vivo* phosphorylation of Nck in HTC-IR cells (Fig. 12B) are superimposable to phosphopeptides b and e from the *in vitro* phosphorylation of GST-3×SH3 of Nck by CKI-γ2 (Figure 11C). The results suggest that Nck is not only a substrate of CKI-γ2 *in vitro* but that it may also be phosphorylated by CKI-γ2 *in vivo*.

Figure 12: *In Vivo* Phosphorylation of Nck

A ^{32}P-Labeled Nck from HTC-IR cells



B Nck Phosphopeptide Map Analysis

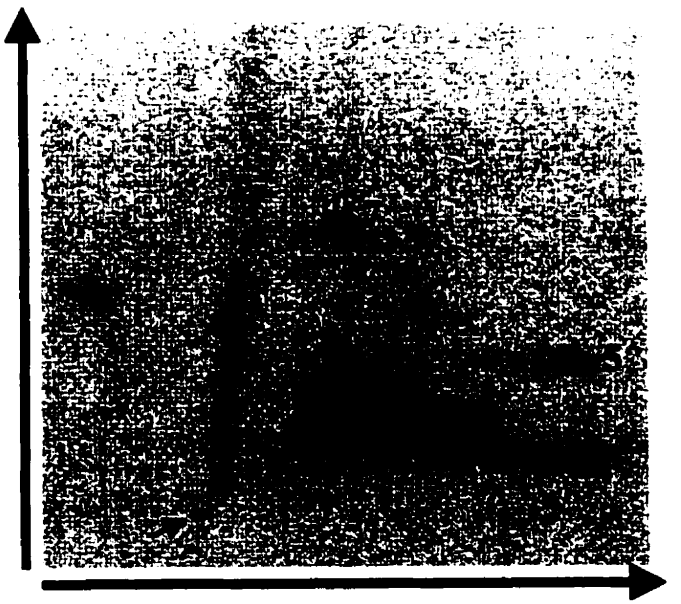


Figure 12: *In Vivo* Phosphorylation of Nck

(A) Subconfluent HTC-IR were metabolically labeled with [^{32}P]-orthophosphate as described in "Materials and Methods". After 4h of cell labeling, [^{32}P]-labeled HTC-IR cell lysates were prepared and immunoprecipitated with anti-Nck antibody (1698). Immunoprecipitated proteins were subjected to SDS-PAGE, transferred to nitrocellulose, and exposed for autoradiography. **(B)** Phosphorylated Nck samples were subjected to proteolytic phosphopeptide mapping as described in "Materials and Methods".

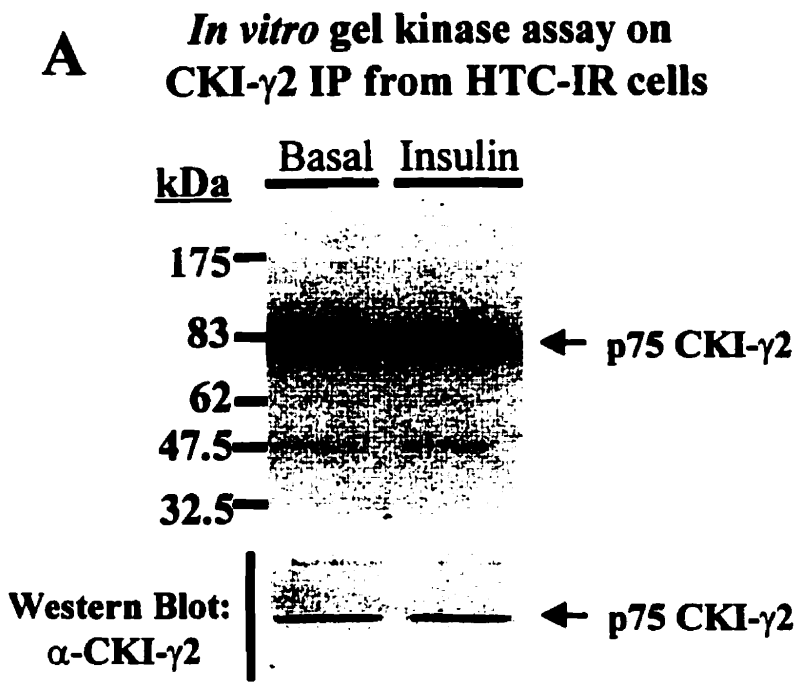
3.3 Objective #3: To determine whether insulin modulates CKI-γ2 activity and consequently, Nck phosphorylation *in vivo*.

3.3.1 Insulin Regulation of CKI-γ2 Kinase Activity

Members of the CKI protein kinase family are poorly understood because of their lack of any detectable regulation by growth factors or hormones in cells. In the insulin signaling network, Nck was reported to associate with IRS-1 in insulin-stimulated cells, suggesting that Nck may participate in mediating insulin actions. The identification of the constitutive association of CKI-γ2 with Nck suggests that CKI-γ2 could be associated to the signaling pathways initiated by the insulin receptor. To investigate whether CKI-γ2 activity is modulated by insulin, lysates from HTC-IR cells treated or not with insulin, were submitted to CKI-γ2 immunoprecipitation. The immunoprecipitated proteins were subjected to an *in vitro* gel kinase assay, a procedure which allows detection of protein kinase in gel by their ability to phosphorylate the exogenous substrate, α-casein, embedded throughout the gel. Upon 5min of insulin stimulation, the phosphorylation of α-casein by CKI-γ2 is reduced by 43.2% as compared to CKI-γ2 from unstimulated HTC-IR cells (Fig. 13A and 13B). Different amounts of immunoprecipitated CKI-γ2 could not account for the decreased activity (Fig. 13A bottom panel). The results therefore suggest that insulin negatively regulates CKI-γ2 kinase activity. In our previous study (96), insulin stimulation did not seem to affect Nck-associated kinase activity towards α-casein including p75 CKI-γ2. However, the *in vitro* gel kinase assay on CKI-γ2 immunoprecipitates is a more sensitive method to detect kinase activity as compared to *in vitro* phosphorylation of α-casein on Nck immunoprecipitates.

To distinguish between total CKI-γ2 and the fraction of CKI-γ2 associated with Nck in cells, Nck was immunoprecipitated from HTC-IR cells treated with or without insulin for various times. The Nck immunoprecipitates were subsequently submitted to *in vitro* gel kinase assays. The kinase activity detected

Figure 13: Insulin Regulation of CKI-γ2 Kinase Activity



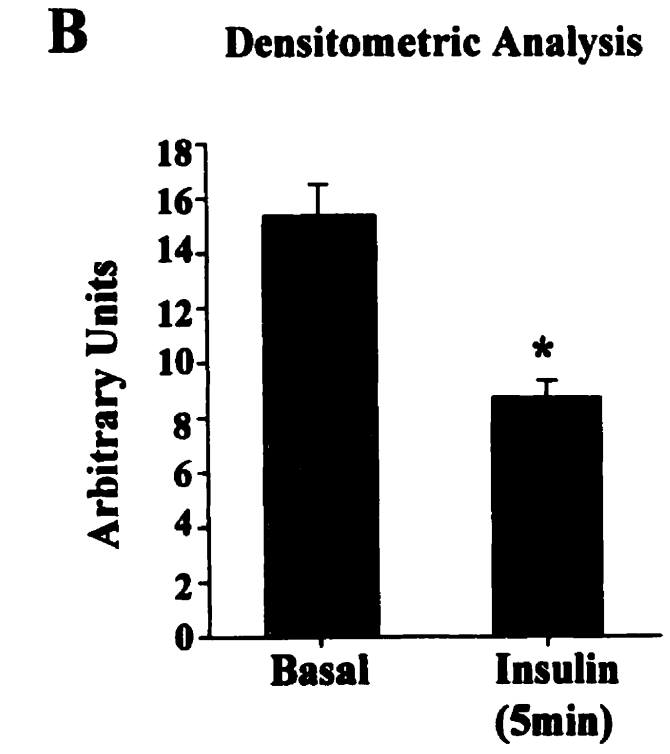


Figure 13: Insulin Regulation of CKI-γ2 Kinase Activity

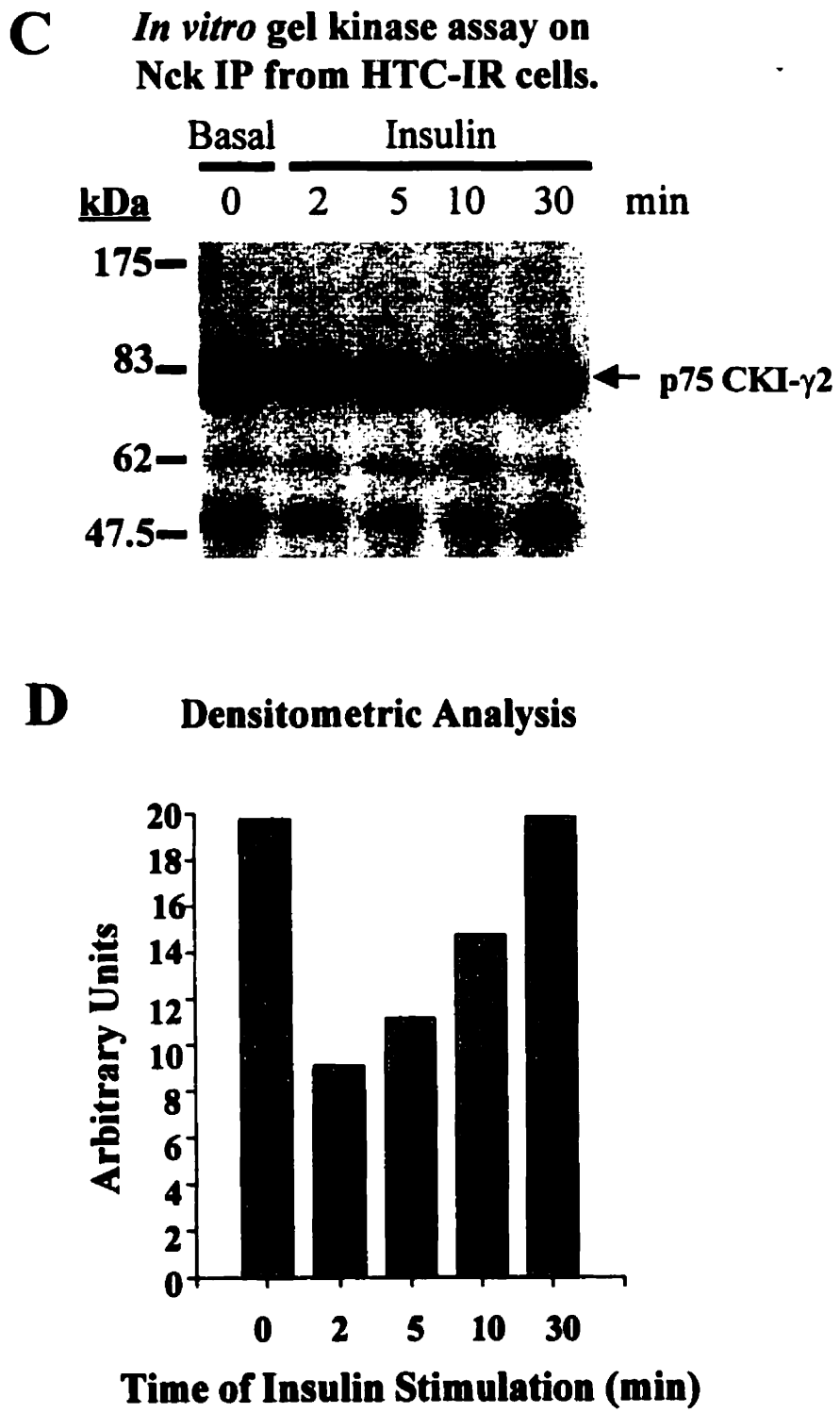


Figure 13: Insulin Regulation of CKI-γ2 Kinase Activity

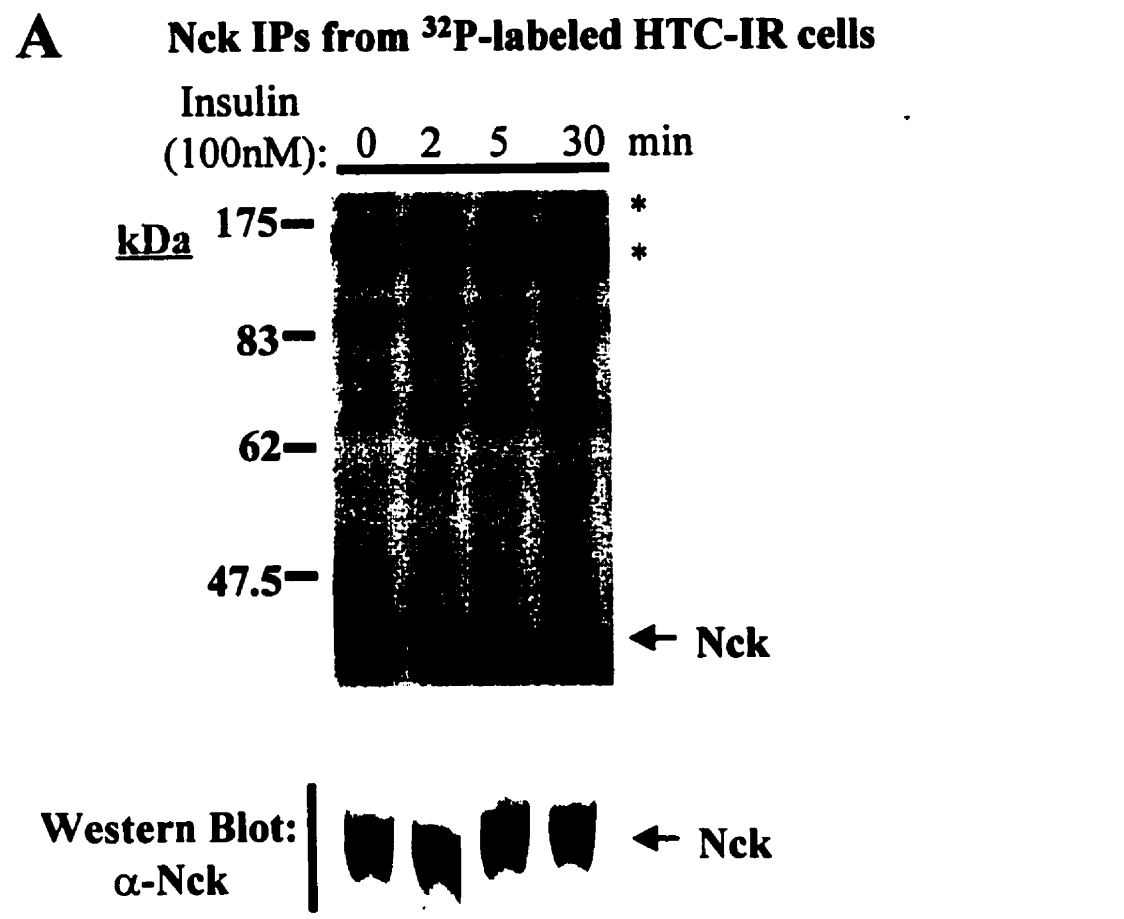
(A) Serum-starved HTC-IR cells were treated or not with insulin (100nM) for 5min. At the end of stimulation, basal and insulin-stimulated cell lysates were prepared in 1% Triton lysis buffer. Samples were immunoprecipitated with anti-CKI-γ2 antibody (1602) and subsequently subjected to *in vitro* gel kinase assays as described in "Materials and Methods"**(top panel)**. CKI-γ2 immunoprecipitates were immunoblotted with anti-CKI-γ2 antibody (1602) as control for the amount of CKI-γ2 protein immunoprecipitated **(bottom panel)**. **(B)** Analysis of results in A by densitometry. The results presented are from three separate experiments (mean ± standard error). *: Data are significant with $p < 0.005$. **(C)** Similarly, serum-starved HTC-IR cells were stimulated with insulin (100nM) for 0, 2, 5, 10, and 30min. Basal and insulin-stimulated cell lysates were immunoprecipitated with anti-Nck antibody (1698) and then subjected to *in vitro* gel kinase assays. **(D)** Analysis of results in C by densitometry (mean ± standard error).

for the p75 CKI-γ2 coimmunoprecipitated with Nck is reduced with insulin stimulation (Fig. 13C). The regulation of CKI-γ2 activity by insulin is a transient phenomena whereby maximal inhibition of CKI-γ2 activity is observed at 2min of stimulation and returns to nearly basal levels by 30min of stimulation.

3.3.2 Effect of Insulin on *In Vivo* Nck Phosphorylation

Since apparently Nck is phosphorylated *in vitro* and *in vivo* by CKI-γ2 and that CKI-γ2 activity is negatively regulated by insulin, the effect of insulin on Nck phosphorylation was examined *in vivo*. HTC-IR cells were labeled with [^{32}P]-orthophosphate and stimulated with insulin during the last 2, 5, or 30min of labeling. Nck, immunoprecipitated from [^{32}P]-labeled HTC-IR cells, showed a constant level of Nck phosphorylation *in vivo*, regardless of insulin stimulation (Fig. 14A top panel). Immunoblotting with anti-Nck antibody (1698) showed that an equal amount of Nck protein was found in all Nck immunoprecipitates (Fig. 14A bottom panel). Phosphoamino acid analysis revealed that Nck phosphorylation from basal or insulin-stimulated cells was solely on serine residues (Fig. 14B). All together, the results suggest that despite decreased kinase activity of CKI-γ2 by insulin, the total phosphorylation level of Nck remains unaltered in cells despite it being a substrate for CKI-γ2. It should be noted that although the level of Nck phosphorylation is unchanged, the negative regulation of CKI-γ2 by insulin may have an effect on either the phosphorylation or association of other proteins found in Nck immunoprecipitates. This is suggested by the concomitant inhibition of the labeling of some proteins in Nck immunoprecipitates upon insulin stimulation (Fig. 14A asterisks and Fig. 13C).

Figure 14: Effect of Insulin on *In Vivo* Nck Phosphorylation



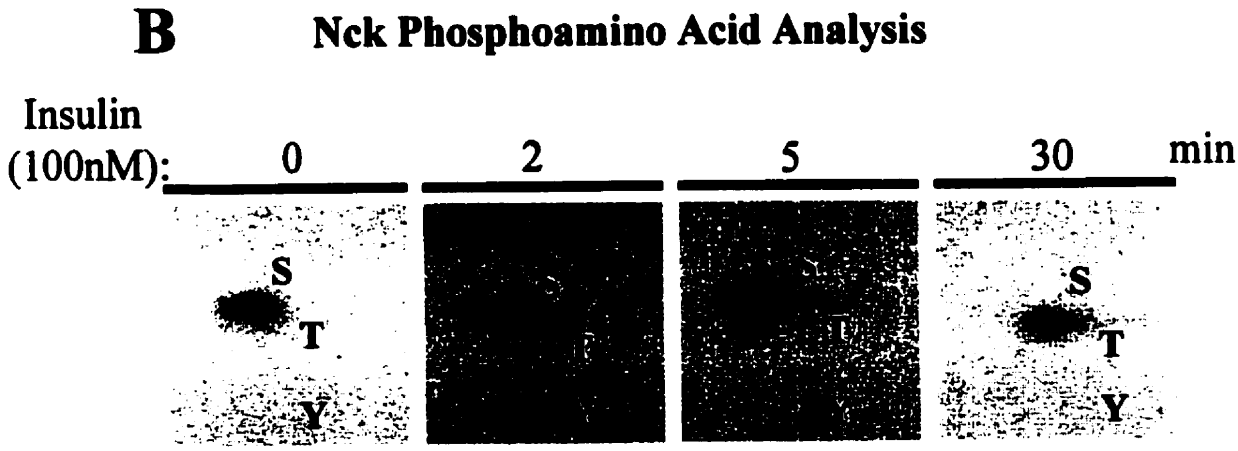


Figure 14: Effect of Insulin on *In Vivo* Nck Phosphorylation

(A) Subconfluent HTC-IR cells were labeled with [^{32}P]-orthophosphate as indicated in figure 13 except that during the last 2, 5, or 30min of labeling, 100nM insulin was added. The clarified HTC-IR cell lysates were immunoprecipitated with anti-Nck antibody (1698). Nck immunoprecipitates were subjected to SDS-PAGE, transferred to PVDF membrane, and exposed for autoradiography **(top panel)**. The amount of Nck protein in each Nck immunoprecipitate was analyzed by immunoblotting with anti-Nck antibody (1698), protein A-HRP, and ECL **(bottom panel)**. **(B)** Phosphorylated Nck samples were subjected to phosphoamino acid analysis as described in "Materials and Methods". S: phosphoserine, T: phosphothreonine, and Y: phosphotyrosine.

Chapter Four

Discussion

Little is known about the CKI family of proteins despite the fact that they are among the first protein kinases discovered. With the exception of CKI-δ and CKI-ε, possible regulation of their activity by hormones, second messengers, or molecular interactions has not been clearly identified. Physiological substrates of CKI are few in number despite the fact that numerous proteins have been identified as substrates *in vitro*. This is a group of enzymes which are ubiquitously expressed in all species but whose biological function is undetermined. This study aimed to learn more about the biological importance of this family of protein kinases by focusing on one of its members, the γ2-isoform (CKI-γ2).

In this study, Northern blot analysis of total RNA prepared from several mouse tissues and cell lines from diverse species, revealed a single ubiquitously expressed CKI-γ2 RNA transcript of 2.4kb. The size of the CKI-γ2 RNA transcript detected here, agrees with the one already reported by other groups (74, 75) and its specificity is supported by the fact that no other transcript related to CKI-γ1 (2.0kb) or CKI-γ3 (2.8kb) (74) was detected. However, ubiquitous expression of the CKI-γ2 RNA transcript was not previously observed by other groups who detected its expression only in poly(A)+ RNA prepared from rat testis. This discrepancy between ubiquitous versus restricted expression of CKI-γ2 RNA transcript may be explained by the use of different probes in all these studies. In fact, from our cloning data, we have found two different species of CKI-γ2 cDNA containing identical coding regions but divergent 3'UTR sequences immediately after the stop codon (CKI-γ2 #9 and CKI-γ2 #14), suggesting that two different CKI-γ2 RNA transcripts could exist. Analysis of both 3'UTR sequences in EST databases revealed that the 3'UTR of clone #14 and not the one of clone #9, was expressed in a wide variety of tissues from different species. For

Northern blot analysis, we have used a cDNA probe derived from the coding region more specific to CKI-γ2 and the other groups used probes derived from the 3'UTR sequence corresponding to clone #9. In these conditions, we believe that we have detected the total population of CKI-γ2 RNA transcripts and in contrast, the other groups may have detected only a subpopulation of transcripts, similar in size but less abundant. This hypothesis is supported by preliminary results obtained from Northern blot analysis on total RNA prepared from Rat2 fibroblasts where only probes from the CKI-γ2 coding region and the 3'UTR of clone #14 have detected a single 2.4kb RNA transcript (data not shown). No signal was found when the 3'UTR sequence of clone #9 was used, suggesting that this species of CKI-γ2 RNA transcript is less abundant, at least in this cell line. It is interesting to note that a similar situation was observed for CKI-δ. Cloning of CKI-δ cDNA has resulted in the isolation of two clones containing identical coding regions but diverging 3'UTR. Northern blot analysis performed with probes specific for each 3'UTR, determined that only one of them represents the predominant form of CKI-δ RNA transcript (76).

CKI-γ2 antibodies used in immunological detection by Western blot of proteins prepared from different murine tissues, revealed that CKI-γ2, is a protein ubiquitously expressed as it was suggested by our Northern blot analysis. Two different polyclonal antibodies, one raised against the C-terminal region of CKI-γ2 and the other raised against the total protein, specifically recognized a single protein of 75kDa as CKI-γ2, and the specificity of these antibodies has been confirmed by their complete displacement by their respective antigen. According to its cDNA, the predicted size for CKI-γ2 is 45.5kDa, however, no protein of this size was detected in any tissues tested, not even in testis where according to our Northern blot analysis and those reported by other groups, the CKI-γ2 RNA transcript was the most highly expressed. This study is the first and the only one, to detect CKI-γ2 as a protein expressed in mammalian tissues and cell lines. For this reason, it is impossible to compare the size that we found for CKI-γ2 with the

findings of other groups. However, when CKI-γ2 is expressed as a recombinant protein in bacteria, or *in vitro* transcribed/translated, the size of the protein produced is in agreement with the one predicted by its cDNA. Interestingly, these protein products were all recognized by both of our polyclonal CKI-γ2 antibodies indicating that if a CKI-γ2 of 45kDa would have existed in murine tissues, our polyclonal CKI-γ2 antibodies would have picked it up. Moreover, transient transfection of the CKI-γ2 cDNA into mammalian cells have shown that in addition to the endogenous p75 CKI-γ2, now the immunological detection of CKI-γ2 reveals a protein of 45kDa with no apparent detectable change in the amount of the immunoreactive p75 CKI-γ2. Although these results do not support that the immunoreactive p75 CKI-γ2 is a result of a post-translational modification of the p45 CKI-γ2, we cannot exclude that such process if it exists, could be rate limited or already saturated. Alternatively, another explanation for the size discrepancy of CKI-γ2 is that the cDNA published for CKI-γ2 is incomplete. However, analysis of the published sequence for rat CKI-γ2 cDNA (74) revealed an upstream stop codon (nucleotide 55) present prior to the start codon (nucleotide 193) in the same reading frame. This confirmed the correct assignment of the initiation codon in CKI-γ2 cDNA with a predicted Mr of 45.5kDa for the protein.

Discrepancies in the size of a protein observed in SDS-PAGE versus the deduced size from the cDNA have been observed for other gene products. For example, the WASP gene product has an observed 13kDa difference from its theoretical size. This anomalous migration in SDS-PAGE is ascribed to its high proline content (18% of total amino acids) (120). Nevertheless, for CKI-γ2 its proline content is only 6% and cannot explain the 30kDa size difference observed. A size discrepancy was also observed for IRS-1 whose predicted molecular mass is 131kDa but migrates as a 165-180kDa protein and this difference was attributed to its hyperphosphorylated state (138). Similarly, CKI-δ and CKI-ε, show approximately 20kDa differences in size due also to their phosphorylation (90). However, hyperphosphorylation of CKI-γ2 does not seem to contribute to its

unexpected molecular weight since *in vitro* treatment of immunoprecipitated CKI-γ2 (p75) with phosphatases does not result in lower molecular weight species of CKI-γ2. Although phosphorylation cannot be excluded completely, it is likely that some other post-translational modification of CKI-γ2 might be responsible for the observed 75kDa molecular weight of the immunoreactive CKI-γ2. For example, RanGAP1 runs as a higher molecular weight protein in SDS-PAGE because it is post-translational modified by the covalent linkage of a ubiquitin-like protein called, SUMO-1 (139, 140). Such unidentified post-translational modification of CKI-γ2 needs to be investigated.

In this study, we have demonstrated that a molecular complex composed of p75 CKI-γ2 and Nck exists in HTC-IR cells. Interestingly, as mRNA and protein, CKI-γ2 and Nck are expressed in the same murine tissues with especially high levels for both in testis. Strong evidence that the p75 immunoreactive CKI-γ2 coimmunoprecipitated with Nck is indeed CKI-γ2 was revealed by the fact that the p75 immunoreactive CKI-γ2 associated with Nck exhibits similar enzymatic characteristics as the recombinant and endogenous immunoprecipitated p75 CKI-γ2. For instance, all three sources of CKI-γ2 demonstrated magnesium-dependent activity as previously reported for recombinant CKI-γ2 (74). Furthermore, CKI-7 inhibits all three sources of CKI-γ2 activity in a range of concentrations where it specifically inhibits CKI enzymes (15, 74). These results provide strong evidence that the three sources of proteins are the same enzyme. Interestingly, CKI-γ2 is the only γ-isoform that contains in its C-terminal domain, PXXP ($_{340}$VHPDVPSQPPHR$_{351}$) consensus motifs for SH3-mediated interaction, supporting its constitutive association with Nck. Although this remains to be proven, it is of interest to find that one of these motifs, Pro-Ser-Glu-Pro, is conserved in human CKI-γ2 as well (75). It is however, important to obtain in future studies, the direct protein sequence of p75 CKI-γ2 coprecipitated with Nck, in order to confirm its identity and to elucidate the post-translational modification responsible for its higher than expected molecular weight. This study provides

strong evidence that immunoreactive p75 CKI-γ2 associated with Nck is indeed CKI-γ2 and furthermore, these findings suggest that CKI-γ2 may participate in signal transduction downstream of RTKs through Nck.

Nck appears to be a substrate of variety of protein tyrosine kinases linked to cell surface receptors and ser/thr kinases activated by growth factors (EGF and PDGF), phorbol esters, and cAMP (99). The fact that Nck is constitutively associated with the ser/thr kinase, CKI-γ2, led us to determine if Nck could be phosphorylated by this enzyme. The results showed that indeed, Nck was an efficient substrate for CKI-γ2 phosphorylation, *in vitro*. On Nck, the sites of CKI-γ2 phosphorylation seem to be localized within Nck's SH3 domains as the three SH3 domains and not the SH2 domain of Nck was phosphorylated. More specifically, CKI-γ2 phosphorylation sites appear to be located in the 2nd SH3 domain of Nck as it was the only domain phosphorylated among the three individual SH3 domains. In fact, two out of three potential CKI-γ2 consensus phosphorylation sites in Nck are found in its 2nd SH3 domain. Similarly, Pak1, a 65kDa ser/thr kinase associated to Nck via its second SH3 domain, also phosphorylates Nck *in vitro* (125, 126). In contrast, Pak1 phosphorylates sites in Nck's SH3#1, SH3#2, SH3#3 and also its SH2 domain (125). The *in vitro* phosphorylation of Nck's SH3 domains by CKI-γ2, shared some of the phosphopeptides obtained from *in vivo* [^{32}P]-labeled Nck isolated from rat HTC-IR and murine NIH 3T3 cells (109). All together these data support the *in vitro* and *in vivo* phosphorylation of Nck by CKI-γ2. Nck mediated signaling downstream of activated RTKs seems to involve its phosphorylation and association with ser/thr kinase such as Pak1 and CKI-γ2. However, in both situations, the significance of these phosphorylations on Nck function has not yet been addressed. Perhaps CKI-γ2 phosphorylation of Nck may create new binding sites for Nck or change its affinity for other Nck-interacting proteins.

Nck is found associated with IRS-1 upon insulin stimulation suggesting that

specific effector molecules bound to Nck could be involved in propagating insulin action. We have investigated whether CKI-γ2 could be regulated by insulin and if so, how CKI-γ2 regulation affects the phosphorylation of Nck. The results show that insulin negatively regulates CKI-γ2 kinase activity in HTC-IR cells. In particular, the fraction of CKI-γ2 associated with Nck, is also negatively regulated by insulin stimulation. Moreover, a time course of insulin stimulation suggests that CKI-γ2 regulation by insulin, is a tightly regulated, transient phenomena. Regulation of CKI activity by the hormone, insulin, may represent a common mechanism of regulation for the poorly understood family of protein kinases. Our results contrast with those from earlier studies, which suggest that, the mammalian CKI is activated by insulin (83). However, in these early studies, CKI was considered a single entity and the different isoforms of CKI enzymes were not known. It has become apparent that the different CKI isoforms have particular enzymatic properties and varying C-terminal extensions, a domain implicated in regulation of the N-terminal catalytic domain. This suggests that different CKI isoforms may have different mechanisms of regulation depending on the length and specificity of their C-terminal domain.

Since Nck was found to be a substrate of CKI-γ2, a kinase likely regulated by insulin, the effect of insulin on Nck phosphorylation was examined. Despite decreased activity of CKI-γ2 by insulin, total phosphorylation level of Nck in HTC-IR cells remains unaltered and solely on serine residues, regardless of time of insulin stimulation. The results agree with previous studies that showed that Nck phosphorylation is solely on serine residues since no tyrosine phosphorylation was observed after insulin stimulation (108). Similarly, p85 and Grb2, which bind IRS-1 and participate in insulin signaling, also fail to undergo tyrosine phosphorylation after insulin stimulation (141, 142). Thus IR doesn't seem to regulate the function of these adaptor molecules by tyrosine phosphorylation. It is likely that Nck binding to IRS-1 alone may modulate activity of CKI-γ2, analogous to the regulation of p110 PI3-kinase by its p85 regulatory subunit. Alternatively, binding of Nck to IRS-1 may simply serve to

relocalize CKI-γ2 within cell to site where it can be in close proximity to its physiological substrates. For example, Grb2 binding to IRS-1 functions to position SOS adjacent to Ras in the plasma membrane (108). Interestingly, in our previous study, insulin stimulation did not seem to affect Nck-associated kinase activity towards α-casein but when looked at the overall *in vitro* phosphorylation of proteins coimmunoprecipitated with Nck, some proteins appeared less phosphorylated after 15-30min of insulin stimulation (96). Similarly, in this study, we find that some proteins coimmunoprecipitated with Nck from *in vivo* [^{32}P]-labeled HTC-IR cells are less phosphorylated after 2min of insulin stimulation. This suggests that insulin regulation of CKI-γ2 may not modulate phosphorylation of Nck itself but may change the phosphorylation and/or association of other Nck-associated proteins.

Interestingly, upon insulin stimulation, CKI phosphorylates and inhibits tyrosine kinase activity of the β-subunit of the IR, suggesting that this type of activity may participate in turning off the activity of the IR following its activation (33). Similarly, a recent study in our laboratory has demonstrated that serine phosphorylation of the ligand-activated β-PDGFR by CKI-γ2 inhibits the receptor's autophosphorylating activity (37). One model that may be proposed is that initial binding of insulin to its receptor results in a transient and time-dependent assembly of a scaffold of proteins to the insulin receptor. Inhibition of CKI-γ2, via a ser/thr phosphatase in this protein complex, would allow the transduction of insulin receptor-mediated signals. Eventually, CKI-γ2 is recovered and could shut down these insulin-mediated events by phosphorylating and inhibiting the receptor activity. Alternatively, IRS-1 could be a target of CKI-γ2 since several consensus motifs for phosphorylation by CKI are present in the amino acid sequence of IRS-1. Interestingly, ser/thr phosphorylation of IRS-1, such as treatment of cells with okadaic acid (ser/thr phosphatase inhibitor), prevents insulin-stimulated glucose transport and is therefore linked to decrease in insulin responsiveness (143). The treatment of okadaic acid in insulin-stimulated cells, increases ser/thr phosphorylation of IRS-1, suggesting a mechanism of

negative feedback on insulin signaling, resulting from ser/thr phosphorylation of IRS-1 (144) perhaps by CKI-γ2. Further studies are required to demonstrate whether this is a biological function of CKI-γ2 in insulin signaling.

Conclusion

This study aimed to learn more about the biological importance of CKI-γ2, a member of the poorly understood Casein Kinase I family of protein kinases. It is the first and only study to report that CKI-γ2 mRNA and protein are ubiquitously expressed in mammalian tissues and cell lines. In addition, we have demonstrated that CKI-γ2 as a protein of 75kDa is complexed with the SH2/SH3-containing adaptor protein, Nck, *in vivo*. CKI-γ2 coprecipitated with Nck presents similar enzymatic properties as recombinant and endogenous CKI-γ2, demonstrating that three sources of protein deal with the same enzyme. In future studies, unidentified post-translational modifications of CKI-γ2 that can explain its higher than expected molecular weight should be investigated. Nevertheless, this study presents strong evidence that that immunoreactive p75 CKI-γ2 associated with Nck is indeed CKI-γ2. Furthermore, our results suggest that Nck-mediated signal transduction pathways downstream of activated receptor tyrosine kinases could implicate CKI-γ2 as an important component. The association of CKI-γ2 with Nck, greatly enhances our knowledge of the different signaling pathways in which the Casein Kinase I may participate.

Identification of CKI-γ2 substrates in future studies will support the role of CKI-γ2 in Nck-mediated signaling downstream of the activated insulin receptor. Of interest, Nck itself is an efficient substrate of CKI-γ2, *in vitro*. Furthermore, this study provides preliminary evidence that Nck is phosphorylated by CKI-γ2 *in vivo*. The finding that Nck is a substrate of CKI-γ2, suggests that Nck function may be regulated by CKI-γ2 ser/thr phosphorylation.

Observations that insulin decreases p75 CKI-γ2 activity provide evidence for negative regulation of CKI-γ2 by this hormone. Whether, modulation by insulin is a common mechanism of regulation for other CKI family members remains to

be determined. Nevertheless, these findings suggest that the complex of CKI-γ2 and Nck could have an important function in insulin-mediated signal transduction. One model is that following insulin binding to its receptor, CKI-γ2 and Nck complex is recruited to IRS-1 via Nck's SH2 domain. This initial binding of insulin to its receptor induces signal transduction and concomitantly results in a transient and time-dependent inhibition of CKI-γ2 activity. Eventually, CKI-γ2 activity is recovered and could now participate in the turn off of insulin-mediated events by phosphorylating and inhibiting the receptor. This is supported by observations that CKI and CKI-γ2 in particular, can phosphorylate and inhibit a number of receptors including the IR ((33), (34), (35), (36), (37), (86)). Alternatively, IRS-1, which contains several consensus motifs for CKI phosphorylation, could be a target of CKI-γ2. Ser/thr phosphorylation of IRS-1, perhaps by CKI-γ2, has been shown to decrease insulin responsiveness ((143), (144)). Therefore, CKI-γ2, through ser/thr phosphorylation of IRS-1 or the IR itself, may be implicated in a mechanism of negative feedback on insulin signaling. Further studies are required to demonstrate whether this is the role of CKI-γ2 in insulin signaling.

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