Characterization of Human Copine III as a Phosphoprotein with Associated Kinase Activity[†,‡]

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ABSTRACT: The copines, first described by Creutz et al. [(1998) *J. Biol. Chem. 273*, 1393−1402], comprise a two C2 domain-containing protein family and are known to aggregate phosphatidylserine membranes in a calcium-dependent manner. No enzymatic function has been attributed to copines yet. Due to a cross-reacting activity of Mikβ1, an antibody to the IL-2Rβ chain, we were able to serendipitously purify, partially microsequence, and clone human copine III. The 5 kb copine III transcript is expressed ubiquitously as determined by a multitissue Northern blot analysis. Phosphoamino acid analysis revealed phosphorylation of copine III on serine and threonine residues. In vitro kinase assays were performed with immunoprecipitated endogenous copine III, chromatography-purified endogenous copine III, and recombinant copine III expressed in *Saccharomyces cerevisiae*. The exogenous substrate myelin basic protein was phosphorylated in all in vitro kinase assays containing copine III immunoprecipitate or purified copine III. A 60-kDa band was observed in corresponding in gel kinase assays with staurosporine-activated cells. Cell lines expressing high levels of copine III protein had correspondingly high kinase activity in copine III antiserum immunoprecipitate. However, the copine amino acid sequences lack the traditional kinase catalytic domain. Therefore, the data suggest copine III may possess an intrinsic kinase activity and represent a novel unconventional kinase family.

Phosphorylation events play a central role in many intracellular signaling pathways, including those of proliferation, differentiation, and apoptosis (*1*, *2*). The number of kinases cloned and characterized has increased dramatically over the past several years. Molecular techniques, including low-stringency hybridization, the polymerase chain reaction (PCR)[1] with degenerate oligonucleotide primers, and sequence alignments have allowed identification of genes that contain a highly conserved classical protein kinase catalytic domain. This domain has been retained in the enzymatic repertoire throughout evolution, from yeast to man. The classical catalytic domain is approximately 300 amino acids long, divided into 12 subdomains, and contains a number of highly conserved amino acids, which have been shown through X-ray crystallography to play a role in ATP binding and phosphotransfer (*3*, *4*).

Recently, several kinases have been described that lack one or more features of the classical protein kinase catalytic domain considered critical for enzymatic function (*5*−*14*). For example, the majority of classical kinases utilize a GXGXXG motif located at the amino terminus of the kinase domain or a P loop to bind ATP. However, the kinase BCR uses a pair of cysteines, and actin−fragmin kinase uses an unidentified sequence for catalysis (*5*, *6*). Moreover, the GXGXXG motif in myosin heavy-chain kinase is located at the carboxyl end of the kinase domain (*7*, *8*). The specific activity of unconventional kinases ranges from 1000-fold less (topoisomerase I) to equal (myosin heavy-chain kinase) that of conventional kinases (*7*−*9*). Whether or not these nontraditional kinases have a similar secondary and tertiary structure to traditional kinases awaits X-ray crystallography studies.

Initial studies in our laboratory of interleukin 2 (IL-2) signal transduction events using the anti-IL-2 receptor β (IL-2Rβ) chain antibody, Mikβ1 (*15*), led to our serendipitous discovery of human copine III (CIII). Ishikawa et al. (*16*) simultaneously cloned KIAA0636, which was later renamed CIII. The copine protein family presently contains seven members and was first described by Creutz et al., who cloned copine I (CI) (*17*−*21*). Copines contain two C2 domains, which are approximately 130-residue motifs, that can bind Ca^{2+}, phospholipids, and inositol polyphosphates (*22*). The biological or enzymatic roles of copines have not been identified. However, Creutz et al. (*17*) suggested they may

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[‡] The nucleotide sequence for human copine III reported in this paper has been submitted to GenBank under accession number AF077226.

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[1] Abbreviations: PCR, polymerase chain reaction; IL-2, interleukin 2; IL-2Rβ chain, interleukin 2 receptor β chain; CIII, copine III; CI, copine I; CVI, neuronal copine; PBS, phosphate-buffered saline; PAS, protein A−Sepharose; TBS, Tris-buffered saline; MBP, myelin basic protein; HH1, histone H1; PAA, phosphoamino acid analysis.

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play a role in membrane trafficking, while Nakayama et al. (*18*) suggested neuronal copine (N-copine, CVI), cloned from the hippocampus of kainate-injected mice, is involved in long-term potentiation of synaptic response. We have biochemically isolated and cloned human CIII (GenBank accession number AF077226) and herein present evidence for the first time that CIII has an associated, and possibly intrinsic, kinase activity.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents. Daudi, Jurkat, K562, U937, and THP-1 cell lines were obtained from the ATCC (Gaithersburg, MD) and cultured in complete medium [RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine]. The THP-1 cell line was grown in the presence of 10^{-7} M β-mercaptoethanol. HL-60 cells were obtained from Dr. Michael Andreef's laboratory.

The IL-2Rβ chain antibody Mikβ1 (IgG2a isotype) was a generous gift from Dr. Mitsuru Tsudo (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). It recognizes the IL-2Rβ chain in immunoprecipitation (1:150 000 dilution) but not in immunoblotting applications. GeneMed Synthesis (San Francisco, CA) synthesized a peptide consisting of the last 18 amino acids of CIII and generated a polyclonal antibody to it from two rabbits. The serum and purified antibody from rabbits 6063 and 6064 were tested and subsequently used in immunoprecipitations (1:150) and immunoblotting (1:2000). Staurosporine and okadaic acid were purchased from Alexis Biochemicals (San Diego, CA).

Protein Purification and Microsequencing of 60-kDa Phosphoprotein Immunoprecipitated by Mikβ1. Twenty liters of Jurkat cell culture at a density of $(1-2) \times 10^6$ cells/mL were harvested, washed in phosphate-buffered saline (PBS), and resuspended in 1% NP-40 lysis buffer (1% NP-40, 10 mM sodium fluoride, 5 mM EDTA, TBS, pH 7.6, 0.2 mM aprotinin, and 1 mM leupeptin). Following a 30-min centrifugation at 16000*g*, the supernatant was desalted on a P6 column (Bio-Rad, Hercules, CA) and applied to a HiLoadQ Sepharose fast-flow column (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with 50 mM NaCl/20 mM Tris, pH 7.5. After the column was washed with the same equilibration buffer, proteins were eluted with a salt gradient of 50−200 mM NaCl. Previous chromatography of the ^{32}P-labeled kinase precipitated by Mikβ1 demonstrated that it elutes at 100 mM NaCl. Conductivity readings identified the 100 mM salt fractions, which were then pooled and electrophoretically separated on a Model 491 Prep Cell 8.5% preparative SDS−polyacrylamide gel (Bio-Rad). Fractions containing proteins in the 60-kDa range were pooled, concentrated to 30 μL with ultrafree 15 and 0.5 centrifugal filter devices (Millipore, Bedford, MA), and separated on an isoelectric focusing gel containing two parts 4−6.5 ampholine (Amersham Pharmacia Biotech) to one part 3−10 ampholytes (Bio-Rad) along with a ^{32}P-labeled Mikβ1 immunoprecipitate labeled in an in vitro kinase reaction. The ^{32}P image alignment with the Coomassie blue stained gel allowed identification of four phosphorylated forms of the kinase recognized by Mikβ1. The spots were excised, combined and washed twice in 1 mL of 50% acetonitrile. The sample was sent to Harvard Microchemistry (Boston, MA) for digestion and mass spectrometric analysis.

Construction of CI and CIII Expression Vectors. Primers (Genosys Biotechnology, Woodlands, TX) were designed to amplify full-length CI and CIII with Jurkat cDNA as a template and included *Hin*dIII and *Not*I sites at the 5′ and 3′ ends of the gene, respectively. CI primers were 5′-GGGAAGCTTCATGGCCCACTGCGTGACC-3′ and 5′-GGGGCGGCCGCTGGGGGGCCTGTGCAGG-3′, and CIII primers were 5′-GGGAAGCTTCGCTGCCCAGTGTGTCACA-3′ (from EST W32758, for mammalian construct) or 5′-GGGAAGCTTATGGCTGCCCAGTGTGTCA-3′ (for yeast construct) and 5′-GGGGCGGCCGCCACTGCTTCTGTTGTTTC-3′ (from EST W02910, for mammalian and yeast constructs). PCR was performed with *Taq* polymerase (Gibco BRL, Grand Island, NY), and amplification conditions for both genes were 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C (CI) or 58 °C (CIII) for 30 s, and 72°C for 1.5 min, followed by a 7 min extension at 72 °C. By standard molecular biology techniques (*23*), CI and CIII were subcloned in frame into the pHM6 and pHB6 expression vectors (Roche-Boehringer Mannheim, Indianapolis, IN), with the addition of an HA tag at the amino terminus and a histidine tag at the carboxyl terminus, and into the pYES2/CT yeast expression vector (Invitrogen, Carlsbad, CA), with the addition of V5 and His tags at the carboxyl terminus.

The *Escherichia coli* strain BL21 was transformed with CI, CIII, or carboxyl terminus of CIII in the pHB6 bacterial expression vector. *Saccharomyces cerevisiae* were transformed with the pYES2/CT vector by itself or containing CIII using the *S.c.* Easy Comp transformation kit (Invitrogen). Optimal CIII protein expression was obained with 18 h of galactose induction, and yeast cells were lysed with acid-washed glass beads as suggested by the manufacturer. Proteins were purified on nickel−agarose (Qiagen) according to the manufacturer's instructions.

Hybridization Analysis. A commercially available multiple tissue northern blot (Clontech, Palo Alto, CA) was probed with a 450 base pair PCR fragment of CIII labeled with [α-^{32}P]CTP (Dupont NEN, Boston, MA) by use of a random hexamer priming kit (Gibco BRL). After the prehybridization in express hybridization solution (Clontech) for 30 min at 68 °C, the blot was incubated for 1 h at 68 °C in express hybridization solution containing the denatured labeled probe. Washes were performed according to the manufacturer's directions.

Immunoprecipitations and Kinase Assays. Cells were washed once in PBS, resuspended in either 1% NP-40 lysis buffer or modified RIPA buffer (1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM sodium fluoride, and TBS, pH 7.6) as indicated in the figure captions, incubated at 4 °C for 20 min, and centrifuged at 16000*g*. All lysates were precleared with appropriately matched isotype control antibodies bound to bovine serum albumin- (BSA-) coated protein A−Sepharose (PAS). Immunoprecipitations with appropriate primary antibodies were carried out overnight at 4 °C, and CIII antigen/antibody complexes were incubated with PAS for 45 min at 4 °C. The beads were washed four times in lysis buffer and once in Tris-buffered saline (TBS), pH 7.5. Samples were analyzed either by Western blot, an in vitro or an in gel kinase assay detailed below. For in vitro kinase assays, beads were resuspended in 20 μL of kinase buffer (25 mM HEPES, 100 mM NaCl, 10 mM $MnCl_2$, and 5 mM $MgCl_2$, pH 7.5) containing 10 μCi of [γ-^{32}P]ATP

(6000 Ci/mmol) and in the cases indicated 2.5 μg of myelin basic protein (MBP) or 2.5 μg of histone H1 (HH1) as well. Samples were incubated at 30 °C for 20 min and eluted by addition of 2× Laemmli buffer and boiling for 5 min.

For in gel kinase assays, cells were treated with 0.5 μM staurosporine, lysed, and immunoprecipitated as above. Precipitates were loaded onto an SDS−10% polyacrylamide minigel containing 0.66 mg/mL MBP substrate and separated at 150 V. Subsequently the SDS was removed from the gel with three 20-min washes in 20% 2-propanol in 50 mM Tris, pH 8. Two 30-min incubations with denaturing buffer (6 M guanidine hydrochloride, 50 mM Tris, pH 8, and 5 mM β-mercaptoethanol) were followed by five washes over 18 h in renaturing buffer (50 mM Tris, pH 8, 5 mM β-mercaptoethanol, and 0.04% Tween-20). The gel was preincubated for 30 min in kinase buffer, and the kinase reaction was carried out in 50 mL of kinase buffer containing 2 μM ATP and 2.5 μCi mL^{-1} [γ-^{32}P]ATP. Unincorporated [^{32}P]ATP was washed out in 5% trichloroacetic acid and 1% sodium pyrophosphate with five changes. Images were scanned with a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and analyzed with ImageQuant Version 1.1 software.

^{35}S Labeling. Daudi cells (2×10^7) were washed twice in long-term labeling medium (met-RPMI, 10% FBS, and 10% 1640 serum-free RPMI). The cells were resuspended in 10 mL of long-term labeling medium containing 1 mCi of [^{35}S]-Met/EXPRE^{35}S^{35}S (Dupont NEN) and incubated 19 h in a humidified incubator set at 37 °C and 7% CO_2. After two washes in ice-cold PBS, cells were resuspended in 1% NP-40 lysis buffer and immunoprecipitated as described above. Following the last wash, 20 μL of 2X Laemmli buffer was added to the samples, and they were boiled for 5 min and analyzed by SDS−PAGE.

CIII Protein Purification. Three liters of Daudi cell culture at a density of $(1-2) \times 10^6$ cells/mL (approximately 5×10^9 cells total) were harvested, washed in PBS, resuspended in 100 mL of lysis buffer, and rotated for 30 min at 4 °C. Following a 30-min centrifugation at 13 000 rpm in an SS34 rotor, the supernatant was dialyzed overnight against 3 L of 20 mM Tris, pH 7.5, with two changes of buffer. The lysate (240 mg) was applied to a HiLoad Q Sepharose fast-flow column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris, pH 7.5. After the column was washed with the same equilibration buffer, proteins were eluted with a salt gradient of 0−400 mM NaCl in 20 mM Tris, pH 7.5. Conductivity readings were taken on all fractions.

Fractions containing CIII (15.6 mg), as determined by Western blot, with corresponding NaCl concentrations between 60 and 180 mM were pooled and loaded onto a CIII affinity column equilibrated with 150 mM NaCl and 20 mM Tris-HCl, pH 7.5. The column was generated from the PAS Kit (Pierce) according to the manufacturer's directions with 10 mg of affinity-purified 6063 CIII antibody and disuccinimidyl suberate as a cross-linker. The column was washed with equilibration buffer and CIII was eluted with elution buffer (50 mM glycine, pH 2.5, 150 mM NaCl, and 0.1% Triton X-100). Fifteen 500-μL fractions were collected in tubes containing 100 μL of 1 M Tris-HCl, pH 9.0, and analyzed by one- and two-dimensional Western and silver staining. Buffer was exchanged to 20 mM Tris-HCl, pH 7.5, with Centricon microfilters prior to in vitro kinase analysis. Approximately 1 μg of protein was recovered from the affinity column.

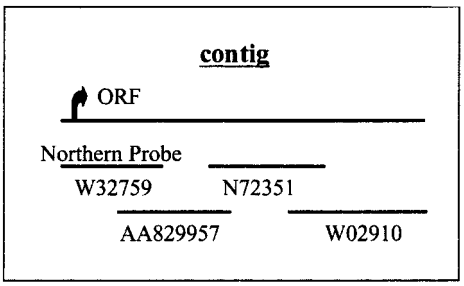

FIGURE 1: Expressed sequence tag (EST) contig. The contig (top line) was obtained by overlapping four EST sequences. The open reading frame (ORF) was determined by searching for a translated protein sequence that was homologous to the 62-kDa human CI.

Phosphoamino Acid Analysis of CIII. Daudi cells (2×10^7) were washed in labeling medium (phosphate-free DMEM and 2% FBS), resuspended in 1 mL of labeling medium and 1 mCi of ^{32}P, and incubated for 2 h at 37 °C. Cells were treated with 1 μM okadaic acid for 45 min prior to 3 washes in ice-cold PBS. Cells were lysed and SDS−PAGE was performed on immunoprecipitates. Proteins were extracted from the gel and phosphoamino acid analysis (PAA) was performed (*24*).

General Methods. All sequencing reactions of copine III in the pHM6 vector were performed with the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech), according to the manufacturer's directions. Protein concentrations were determined with the DC (detergent compatible) protein assay kit (Bio-Rad), following manufacturer's instructions. Western blotting procedures consisted of a 1-h incubation in blocking solution (5% nonfat dry milk, PBS), a 1.5-h incubation with the appropriate primary antibody, three 5-min washes in PBS containing 0.1% Tween-20, a 45-min incubation with secondary antibody (1:4000 goat anti-mouse or rabbit) in PBS containing 5% milk and 0.1% Tween-20, and three 5-min washes in PBS containing 0.1% Tween-20. The blots were developed with ECL reagent (Amersham Pharmacia Biotech). The silver stain was performed with a modified version of the monochromatic silver stain (*25*).

RESULTS

Cloning. Experiments were designed to identify kinases involved in signal transduction, as natural killer cells are activated by IL-2 to acquire LAK (lymphokine activated killing) activity. Immunoprecipitations with the monoclonal antibody Mikβ1, followed by in vitro and in gel kinase assays, identified an unknown 60-kDa phosphoprotein/kinase. Mikβ1 was also found to immunoprecipitate this protein from the intended negative control of the IL-2Rβ negative Jurkat cell line; therefore, this antibody was proposed to recognize the 60-kDa protein directly. Mikβ1 was subsequently employed in a purification scheme to identify the 60-kDa kinase by 2D gel analysis. The 60-kDa protein spots were excised, digested, and quantitated, and the resulting peptides were separated by high-performance liquid chromatography.

Five peptides obtained from mass spectrometric analysis were used to screen databases by use of the BLASTP algorithm (*26*), but no full-length gene matches were found. One peptide, SSPVEFECINEK, was then used to screen a

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5′ UTR  CTAAGACTCCCACGAAACTCAGGTTGAATAATTCATCAAATTACACAACTGAACTCAAGAC

 M   A   A   Q   C   V   T   K   V   A   L   N   V   S   C   A   N   L   L   D
ATG GCT GCC CAG TGT GTC ACA AAG GTG GCG CTG AAT GTT TCC TGT GCC AAT CTT TTG GAT
 K   D   I   G   S   K   S   D   P   L   C   V   L   F   L   N   T   S   G   Q
AAA GAT ATA GGG TCA AAG TCA GAC CCT TTA TGT GTG TTG TTT TTG AAT ACA AGT GGT CAA
 Q   W   Y   E   V   E   R   T   E   R   I   K   N   C   L   N   P   Q   F   S
CAG TGG TAT GAG GTT GAG CGC ACA GAA AGG ATT AAG AAT TGC TTG AAT CCC CAA TTT TCC
 K   T   F   I   I   D   Y   Y   F   E   V   V   Q   K   L   K   F   G   V   Y
AAG ACA TTT ATT ATT GAT TAC TAC TTT GAA GTG GTT CAG AAA TTG AAA TTT GGG GTT TAT
 D   I   D   N   K   T   I   E   L   S   D   D   D   F   L   G   E   C   E   C
GAC ATC GAC AAC AAA ACT ATT GAG CTG AGT GAT GAT GAC TTC TTA GGG GAA TGT GAA TGT
 T   L   G   Q   I   V   S   S   K   K   L   T   R   P   L   V   M   K   T   G
ACC CTT GGA CAA ATT GTT TCC AGC AAG AAG CTA ACT CGA CCA CTG GTG ATG AAA ACT GGC
 R   P   A   G   K   G   S   I   T   I   S   A   E   E   I   K   D   N   R   V
AGA CCT GCA GGA AAA GGG AGC ATT ACG ATT TCA GCT GAA GAA ATA AAA GAT AAT AGA GTG
 V   L   F   E   M   E   A   R   K   L   D   N   K   D   L   F   G   K   S   D
GTC TTG TTT GAA ATG GAA GCC AGA AAA CTG GAT AAT AAG GAT CTA TTT GGA AAG TCA GAC
 P   Y   L   E   F   H   K   Q   T   S   D   G   N   W   L   M   V   H   R   T
CCA TAC CTG GAA TTC CAC AAG CAG ACA TCT GAT GGA AAC TGG CTA ATG GTT CAT CGG ACA
 E   V   V   K   N   N   L   N   P   V   W   R   P   F   K   I   S   L   N   S
GAG GTT GTT AAA AAC AAC TTG AAT CCT GTT TGG AGG CCT TTC AAG ATC TCT CTT AAC TCA
 L   C   Y   G   D   M   D   K   T   I   K   V   E   C   Y   D   Y   D   N   D
CTG TGT TAC GGA GAT ATG GAC AAA ACC ATT AAG GTG GAG TGT TAT GAT TAT GAC AAT GAT
 G   S   H   D   L   I   G   T   F   Q   T   T   M   T   K   L   K   E   A   S
GGG TCA CAT GAT CTC ATT GGA ACA TTT CAG ACC ACC ATG ACA AAA CTG AAA GAA GCC TCC
 R   S   S   P   V   E   F   E   C   I   N   E   K   K   R   Q   K   K   K   S
AGA AGC TCA CCT GTT GAA TTT GAA TGC ATA AAT GAG AAA AAA AGG CAA AAG AAA AAA AGC
 Y   K   N   S   G   V   I   S   V   K   Q   C   E   I   T   V   E   C   T   F
TAC AAG AAT TCA GGT GTT ATC AGT GTG AAA CAG TGT GAG ATT ACA GTA GAA TGC ACA TTC
 L   D   Y   I   M   G   G   C   Q   L   N   F   T   V   G   V   D   F   T   G
CTT GAC TAT ATA ATG GGA GGA TGT CAG CTG AAT TTT ACT GTG GGA GTG GAC TTC ACT GGC
 S   N   G   D   P   R   S   P   D   S   L   H   Y   I   S   P   N   G   V   N
TCC AAT GGT GAC CCA AGG TCT CCA GAC TCC CTT CAT TAC ATC AGC CCC AAT GGC GTT AAT
 E   Y   L   T   A   L   W   S   V   G   L   V   I   Q   D   Y   D   A   D   K
GAG TAT TTG ACT GCT CTC TGG TCT GTG GGA CTG GTC ATT CAA GAT TAT GAT GCT GAT AAG
 F   N   P   S   N   P   Y   C   N   G   I   Q   G   I   V   E   A   Y   R   S
ATG TTT CCA GCT TTT GGT TTT GGC GCT CAG ATA CCT CCT CAG TGG CAG GTA TCA CAT GAA
 C   L   P   Q   I   K   L   Y   M   F   P   A   F   G   F   G   A   Q   I   P
TTT CCA ATG AAC TTC AAC CCA TCC AAT CCC TAC TGC AAT GGA ATC CAA GGC ATT GTA GAG
 P   Q   W   Q   V   S   H   E   F   P   M   N   G   P   T   N   F   S   P   I
GCG TAT CGG TCT TGT CTT CCT CAG ATA AAA CTC TAT GGA CCA ACT AAT TTT TCT CCA ATC
 I   N   H   V   A   R   F   A   A   A   A   T   Q   Q   Q   T   A   S   Q   Y
ATA AAT CAC GTG GCC AGG TTT GCT GCT GCA GCC ACG CAA CAG CAG ACA GCT TCT CAA TAT
 F   V   L   L   I   I   T   D   G   V   I   T   D   L   D   E   T   R   Q   A
TTT GTG CTT TTG ATT ATT ACT GAT GGT GTG ATC ACA GAC CTT GAT GAA ACC AGA CAA GCT
 I   V   N   A   S   R   L   P   M   S   I   I   I   V   G   V   G   G   A   D
ATA GTT AAT GCC TCC AGG CTG CCT ATG TCC ATC ATA ATT GTT GGA GTT GGA GGT GCT GAC
 F   S   A   M   E   F   L   D   G   D   G   G   S   L   R   S   P   L   G   E
TTC AGC GCC ATG GAG TTT CTG GAT GGT GAT GGT GGA AGT CTC CGC TCC CCA TTG GGC GAA
 V   A   I   R   D   I   V   Q   F   V   P   F   R   Q   F   Q   N   A   P   K
GTG GCC ATC AGA GAT ATT GTC CAG TTT GTG CCT TTC AGA CAG TTC CAG AAT GCT CCA AAA
 E   A   L   A   Q   C   V   L   A   E   I   P   Q   Q   V   V   G   Y   F   N
GAA GCA CTT GCT CAG TGT GTC TTG GCA GAG ATT CCC CAG CAG GTG GTG GGC TAC TTC AAT
 T   Y   K   L   L   P   P   K   N   P   A   T   K   Q   Q   K   Q
ACA TAC AAA CTC CTT CCT CCC AAG AAC CCA GCC ACG AAA CAA CAG AAG CAG TGA
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FIGURE 2: Nucleotide and deduced amino acid sequence of human CIII. The cDNA sequence was submitted to GenBank (accession number AF077226). Peptides obtained from microsequencing are underlined. Sequence prior to ATG site is 5′-untranslated DNA contained in EST W32759.

nonredundant GenBank EST database with the TBLASTN 2.0.3 algorithm (*26*) and identified W32759, a 443 nucleotide subject EST sequence. When W32759 was used as a query to search the nonredundant GenBank database, the subject sequence with the highest degree of homology was human CI (accession number U83246). Several EST sequences had been assigned to be putative copines II−V by Creutz et al. (*17*), and EST N72351, in particular, had been named CIII. Further database searches for ESTs that would overlap W32759 allowed for the construction of a contig, which contained the entire coding region of CIII (Figure 1). Figure 2 shows the full-length coding CIII cDNA and the predicted amino acid sequence, which were submitted by us to GenBank and assigned accession number AF077226. The chromosomal location of the CIII gene is 8q21 (*16*).

Sequence Analysis of Copine Family. Alignment of the amino acid sequences of CI, CIII, copine VI (N-copine) (CVI), and copine VII reveals several common features

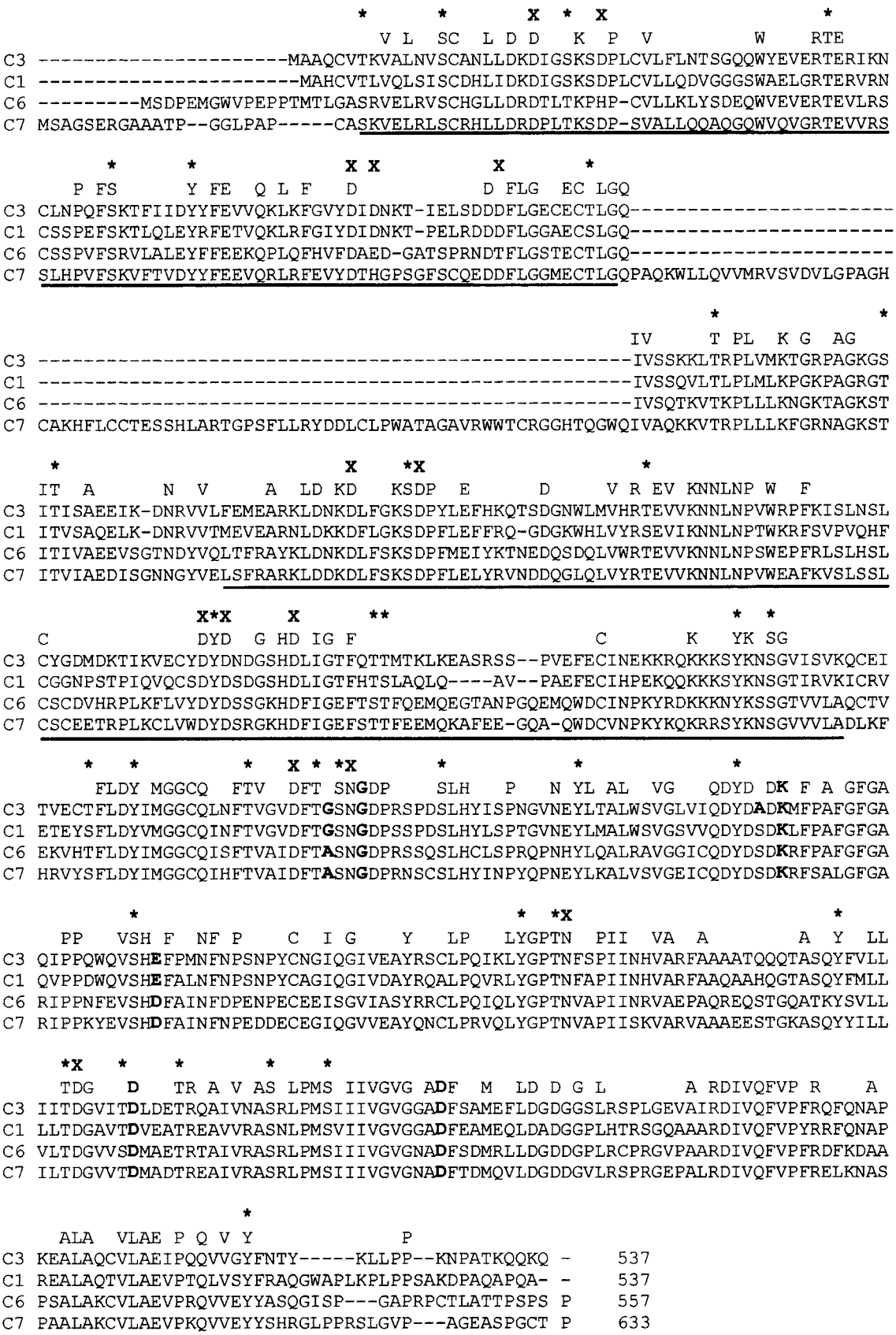

FIGURE 3: Protein sequence alignment of human CI, CIII, CVI, and CVII. The full-length amino acid sequences of human CIII (3), CI (1), CVI (6 or N-copine), and CVII (7) were aligned with MAP software. The two C2 domains at the amino-terminal half of the proteins are underlined. Residues identical in all four copines are shown above CIII. Serines, threonines, and tyrosines conserved in all four proteins are marked with asterisks, indicating possible phosphorylation sites. Proposed calcium (C2 domains) and magnesium (A domain) chelating residues are marked with an X. Conserved residues found in the classic kinase catalytic domain are in boldface type.

(Figure 3). CIII is 63% identical to CI, 52% to CVI, and 47% to copine VII (CVII). All four copines have two C2 domains at their amino termini. The last 25 amino acids at the carboxyl termini of the three copines are highly divergent,

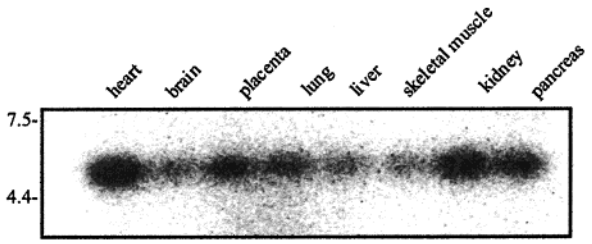

FIGURE 4: Tissue distribution of human CIII mRNA: Multitissue Northern blot analysis of CIII expression with a ^{32}P-labeled fragment containing partial C2 domains of CIII, nucleotides 29−442, as a probe.

and copine VII has a 70 amino acid insert between the two C2 domains. A motif search of CIII against PROSITE with ScanProsite did not reveal the classical kinase catalytic domain (*27*). However, the carboxyl-terminal half of copines has several stretches of identical amino acids that could be critical for copine function.

CIII mRNA Expression. To determine the tissue distribution of CIII message, a multitissue Northern blot was performed with the PCR product of W32759 as a probe. Figure 4 shows that CIII message was detected in all tissues tested. Even though the coding sequence of CIII is only 1614 nucleotides long, the only band detected in the Northern blot was approximately 5 kilobases. The size of this transcript is, however, in agreement with the length of the DNA sequence (4737 nucleotides) for the KIAA0636 protein (AB014536) (*13*).

Characterization of Phosphotransferase Activity Immunoprecipitated with Endogenous CIII. The anti-CIII antibody was tested for its ability to immunoprecipitate CIII and recognize CIII in immunoblots. Figure 5A demonstrates that the CIII immune serum was able to immunoprecipitate ^{35}S-labeled CIII (60 kDa) while the preimmune serum was not. The specificity of the anti-CIII polyclonal antibody was tested against recombinant CI and CIII expressed in *E. coli*. In a Western blot (Figure 5B) the antiserum recognized the denatured form of full-length CIII and its carboxyl terminus but not CI.

In vitro and in gel kinase assays were performed to test whether phosphotransferase activity was associated with CIII. Kinase activity was measured by comparing levels of MBP phosphorylation. As demonstrated in Figure 5C, kinase activity was associated with anti-CIII serum immunoprecipitate from Daudi and K562 lysates but not with preimmune serum.

To determine the size of the kinase immunoprecipitated with the anti-CIII antibody, an in gel kinase assay was performed with staurosporine-activated K562 and Daudi cell lysates. A 60-kDa protein, corresponding to the size of CIII, which was able to phosphorylate MBP in an in gel kinase assay (shown in Figure 5D) was immunoprecipitated from both cell lines with anti-CIII serum. The preimmune serum did not immunoprecipitate a kinase at any molecular weight in staurosporine-activated lysate.

Kinase Activity Associated with Purified Endogenous CIII. An alternative experiment to determine whether copines possess intrinsic kinase activity was to perform an in vitro kinase assay on active endogenous CIII purified to homogeneity. A purification scheme, which included ion-exchange chromatography and affinity chromatography as the two

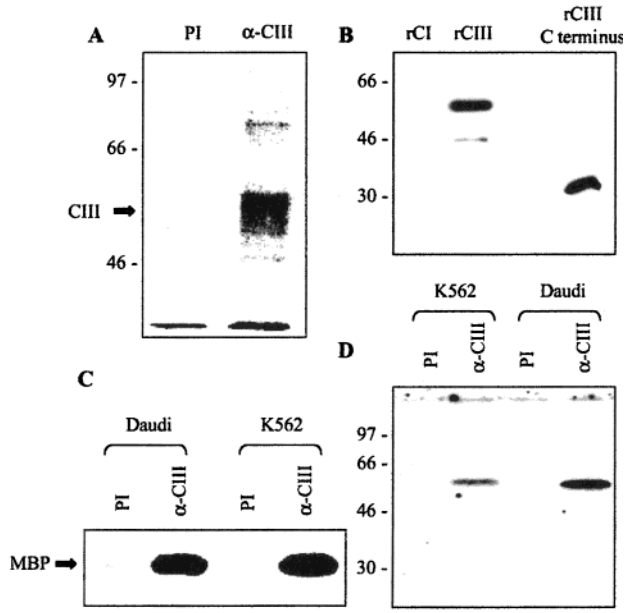

FIGURE 5: Kinase activity associated with endogenous CIII. ^{35}S-labeled Daudi lysate was immunoprecipitated with preimmune (PI) or anti-CIII antiserum (α-CIII) in panel A. Anti-CIII antiserum was used to probe a blot containing recombinant CI, CIII, and CIII C-terminus grown in *E. coli* in panel B. Daudi and K562 cells (10^7 each) were lysed in RIPA buffer, precleared, and immunoprecipitated with PI or α-CIII. Samples were subjected to either an in vitro kinase assay (panel C) or an in gel kinase assay (panel D) with MBP as an exogenous substrate. Cells were treated with 1 μM staurosporine for 45 min prior to lysis (panel D).

major steps, was designed to isolate CIII from Daudi cells. The antibody specific for CIII facilitated the identification of ion-exchange chromatography fractions containing CIII by Western blotting and the generation of a CIII affinity column.

All ion-exchange chromatography fractions were analyzed by Western blot (data not shown). The fractions containing CIII were pooled and separated on the CIII affinity column, and fractions collected from this column were analyzed by Western blot, silver-stained, and tested for phosphotransferase activity. As seen in Figure 6A, fraction 4 contains the highest amount of CIII as determined by Western blot. Fractions 1 and 2 contain no detectable levels, while fractions 5−8 contain progressively lower amounts of CIII than fraction 4. A sample from the pooled ion-exchange fractions containing CIII (lane L) contained CIII at 60-kDa as well as another protein at 70-kDa. This larger protein is consistently recognized by the anti-CIII antiserum in a number of cell lines tested. It could be a larger molecular weight copine family member with a similar carboxyl terminus to CIII or an unrelated protein that merely shares an epitope with CIII. The silver stain of the affinity fractions (Figure 6A) parallels the Western blot results in that fraction 4 contains the highest amount of CIII. Fractions 3, 5, and 6 also contain detectable levels of the 60-kDa CIII.

All of the affinity fractions were tested for the presence of kinase activity by an in vitro kinase assay with HH1 as an exogenous substrate. Figure 6C shows a correlation between kinase activity and the amount of CIII detected by Western blotting of the affinity fractions (Figure 6B). Fraction 4 contained the highest levels of CIII protein, as well as the highest kinase activity, as HH1 is most heavily

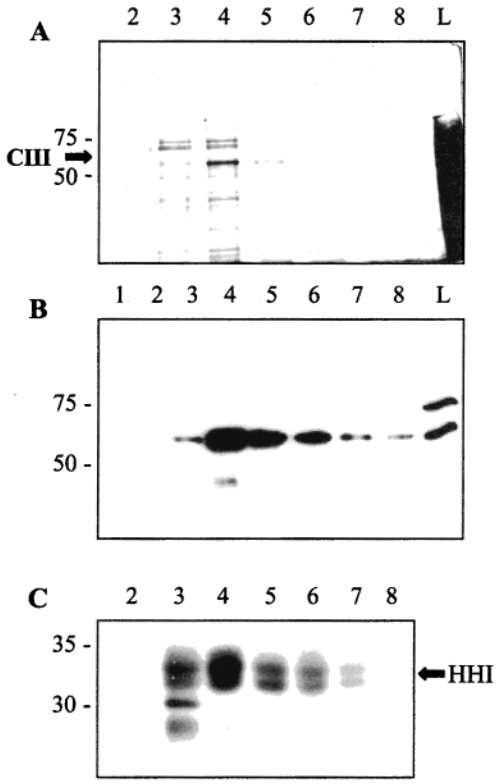

FIGURE 6: Analysis of CIII affinity chromatography fractions. Fractions collected from the CIII affinity chromatography column were analyzed by silver stain after electrophoresis (panel A) and by anti-CIII Western blotting (panel B). L refers to combined sample loaded onto the affinity column. Kinase activity of purified CIII was tested with the exogenous substrate HH1 by an in vitro kinase assay (panel C). Lanes refer to fraction numbers.

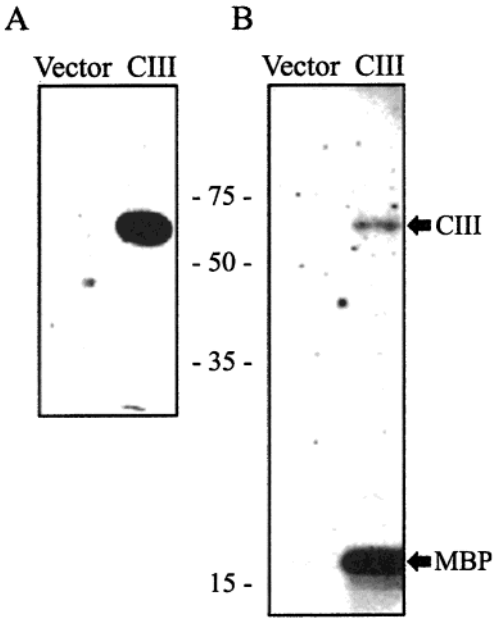

FIGURE 7: Expression and kinase activity of recombinant human CIII expressed in yeast. (A) Five microliters of the nickel−agarose elution 1 from either vector control or CIII-transformed yeast were immunoprecipitated with 2 μL of anti-V5 antibody (Invitrogen) in RIPA buffer. The Western blot was probed with anti-CIII antibody. (B) An in vitro kinase assay with MBP as a substrate was performed on V5 immunoprecipitate from 100 μL of nickel−agarose elution 2 from either vector control or CIII-transformed yeast.

phosphorylated by a kinase in this fraction. Fractions 5 and 6 have no detectable contaminants by silver stain but still retain kinase activity. Preliminary studies indicate that the specific activity of the purified CIII preparation was in the femtomoles per minute per milligram range. A more accurate determination would require larger quantities and use of a natural substrate.

Kinase Activity of Recombinant Human CIII. Recombinant human CIII expressed in *E. coli* was unable to phosphorylate either MBP or HH1 in an in vitro kinase assay (data not shown). Copines are poorly soluble when expressed in *E. coli*, as only about 5% of CIII was located in the soluble fraction, and this may not be folded and phosphorylated correctly to be biologically active. However, copine III containing a V5 and His tag could be expressed at adequate levels in *S. cerevisiae*. Partially purified CIII eluted from nickel−agarose beads was immunoprecipitated with a V5 antibody and was detected by Western blot in Figure 7A. Phosphotransferase activity of the V5 immunoprecipitates from vector control and CIII nickel−agarose eluates were tested in an in vitro kinase assay with MBP as a substrate. As shown in Figure 7B, MBP was strongly phosphorylated by CIII V5 immunoprecipitate as compared to vector control. A band at 65-kDa, corresponding to CIII plus V5/His tag, was also observed, suggesting low levels of autophosphorylation of CIII.

Correlation of CIII Protein Levels and Associated Kinase Activity. CIII protein levels from human tumor cell lines of different lineages, including monocytic (U937, HL-60, THP-1), megakaryocytic (K562), and B cell lymphoma (Daudi), were determined by Western analysis. In Figure 8A, K562 and Daudi expressed high levels of CIII (60-kDa band), while U937, HL-60, and THP-1 express low to undetectable CIII protein. In addition, CIII could not be detected in HL-60 by 1D and 2D Western blot analysis of anti-CIII immunoprecipitate or 2D gel analysis of anti-CIII immunoprecipitate of ^{35}S-labeled cell lysate (data not shown). Therefore, while low levels of CIII can be detected in HL-60 lysate, CIII cannot be immunoprecipitated with our polyclonal antibody to CIII. A prominent band at 70 kDa, which had been detected in previous Westerns, was recognized by the CIII antiserum in most cell lysates. Phorbol 12-myristate 13-acetate treatment or lysis in the presence of 5 mM calcium did not affect CIII protein levels in the cytoplasmic fraction as detected by Western blot (data not shown).

The relative phosphotransferase activity associated with anti-CIII antibody immunoprecipitate was determined by an in vitro kinase with MBP as a substrate. When MBP phosphorylation of preimmune serum was compared with CIII antiserum immunoprecipitate in Figure 8B, only K562 (lane 8) and Daudi (lane 10) samples had high levels of MBP phosphorylation. Thus the amount of kinase activity correlated well with the levels of CIII expression and the ability to immunoprecipitate CIII from cell lysate.

Phosphorylation Status of CIII. To determine if CIII is phosphorylated, CIII was analyzed by 2D Western and PAA. Multiple horizontal spots on 2D gels may indicate the presence of phosphorylated forms of a protein. The anti-CIII antiserum detected three distinct large spots at the same molecular weight but different isoelectric points and one very faint spot on the far left (Figure 9A). This pattern was consistently observed in 2D immunoblots of endogenous

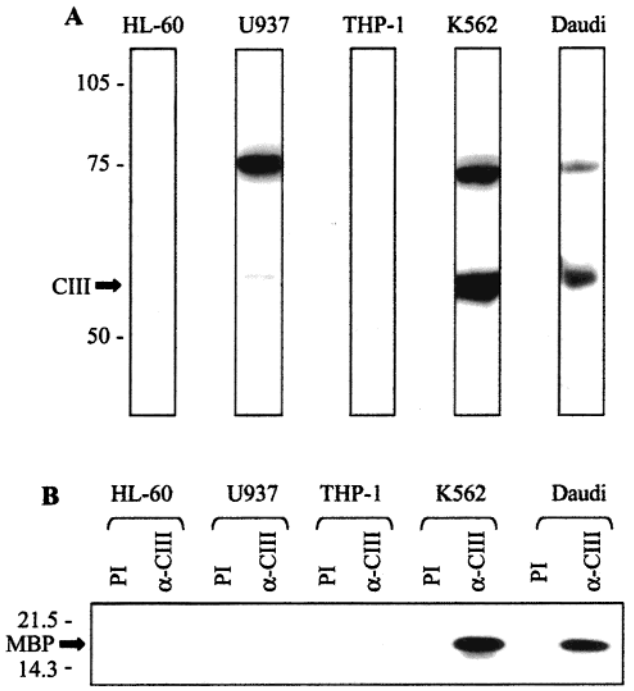

FIGURE 8: Expression of CIII protein and associated kinase activity in a panel of cell lines. (A) Cells were lysed in RIPA buffer. After centrifugation and determination of protein concentration, 100 μg of cleared cell lysate was analyzed by Western blot with anti-CIII serum. (B) An in vitro kinase assay was performed on CIII antibody immunoprecipitate from 500 μg of protein derived from cell lines HL-60 (lanes 1 and 2), U937 (lanes 3 and 4), THP-1 (lanes 5 and 6), K562 (lanes 7 and 8), and Daudi (lanes 9 and 10). The first sample from each set is immunoprecipitated with preimmune serum and the second with anti-CIII antiserum.

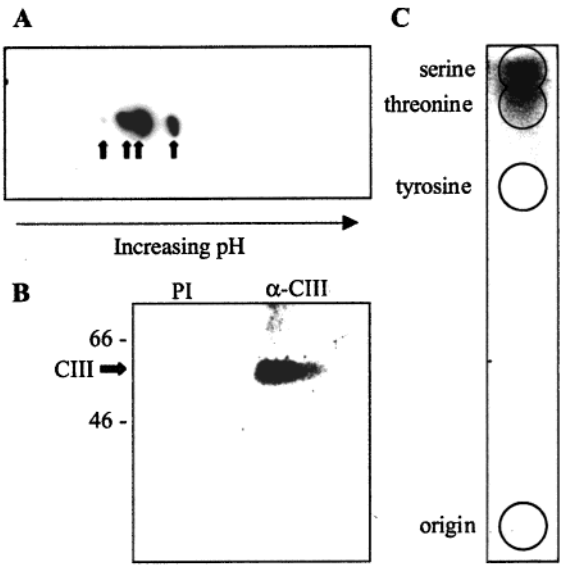

FIGURE 9: In vivo phosphorylation of CIII. (A) Affinity fraction 4 (10 μL) was separated on a 2D gel, transferred to PVDF membrane, and probed with anti-CIII antiserum. [^{32}P]Orthophosphate-labeled CIII was excised from the polyacrylamide gel in panel B and analyzed by phosphoamino acid analysis in panel C.

CIII. The predicted isoelectric point of CI and CIII is close to 6, while the observed p*I* of endogenous CIII ranges from 5 to 5.4. Recombinant CIII expressed in bacteria migrated further toward the basic end of the gel than CIII immunoprecipitated from Daudi cell lysate in a 2D Western blot (data not shown).

PAA was performed on ^{32}P-labeled CIII immunoprecipitated from orthophosphate-labeled Daudi. As demonstrated in Figure 9B, the 60-kDa CIII, is phosphorylated in vivo. According to the PAA (Figure 9C), CIII is phosphorylated on both serine and threonine but not tyrosine residues. All serine, threonine, and tyrosine residues conserved in all four sequenced copines are indicated with asterisks in Figure 3 and could serve as potential phosphate acceptor sites. Therefore, both sequence and biochemical evidence support the idea that copines may be a multiphosphorylated protein family.

DISCUSSION

We have presented experimental evidence that suggests CIII has associated kinase activity and is perhaps itself a bona fide kinase. In vitro assays demonstrate that kinase activity was associated with endogenous CIII immunoprecipitate and with recombinant human CIII expressed in yeast. The phosphotransferase activity of the recombinant CIII in yeast strongly suggests that CIII has intrinsic kinase activity, especially since yeast do not have copine homologues and it is therefore less likely for another kinase to be copurified with the recombinant CIII protein. Phosphorylation of the exogenous substrate MBP by a 60-kDa protein in the in gel kinase assay suggests that the 60-kDa endogenous CIII contains phosphotransferase activity.

Second, the amount of kinase activity (Figure 6) detected in fractions containing purified CIII from the CIII affinity chromatography parallels levels of CIII protein observed in the Western analysis. This indicates either that an active kinase is copurified with CIII or that the kinase is CIII itself. Although it could be argued that some of the MBP phosphorylation is due to one of the contaminating proteins observed in the silver-stained gel (Figure 6), most contaminating proteins were eluted by affinity chromatography fraction 4, while the kinase activity continued up to and including fraction 7, which still contained CIII (Figure 6).

A third set of corroborating evidence for CIII associating with a kinase or possessing intrinsic kinase activity is provided by the correlation between CIII protein levels and kinase activity immunoprecipitated with the anti-CIII antibody. Therefore, biochemical data strongly supports CIII as a kinase, but the possibility of an associated kinase cannot be entirely excluded.

The low specific activity (femtomole per minute per milligram levels) of the purified CIII may be due to several factors. A likely explanation is that MBP and HH1 are not the natural substrates of copines, and as this report is the first suggesting kinase activity associated with copines, CIII substrates are currently unknown. In other systems, myosin heavy-chain kinase A for example, the calculated specific activity was 2.2 and 3.8 mmol min^{-1} mg^{-1} for a specific peptide (MH-3) and MBP, respectively, while histone III-S and histone VIII-S were poor substrates (*7*). Also, copines may require associated proteins or factors for optimal activity. Such factors may not be present in yeast or may have been lost during the purification procedure of the endogenous CIII, resulting in lower kinase activity than in immunoprecipitates of endogenous CIII.

Since a search against PROSITE with ScanProsite on the Expasy site for protein motifs of the CI and CIII protein

sequences did not identify the classical kinase catalytic domain, an additional manual search for conserved residues was performed. The carboxyl termini of CI, CIII, CVI, and CVII aligned in Figure 3 were chosen because the amino termini had already been shown to contain two C2 domains. There are 266 amino acid residues comprising the carboxyl terminus immediately following the second C2 domain. This is within the range of the length of most kinase domains (250−300). Briefly, conserved features of the traditional kinase catalytic domain include 12 subdomains, containing the 12 nearly invariant residues G50 and G52 (subdomain I), K72 (subdomain II), E91 (subdomain III), D166 and N171 (subdomain VIB), D184 and G186 (subdomain VII), E208 (subdomain VIII), D220 and G225 (subdomain IX), and R280 (subdomain XI). The numbering of conserved residues is based on the PKA catalytic subunit α (*28*).

The entire GXGXXG motif, located in subdomain I in the classic kinase domain, is not found in copines. However, there is a glycine residue, conserved in copines I, III, VI, and VII, 32 amino acids downstream of the second C2 domain. Preceding this residue and separated by two residues is another G in CI and CIII and an A in CVI and CVII. Alanines can sometimes substitute for glycines because both amino acids are very small and would not disrupt secondary protein structure. In fact, the glycine loop in NinaC kinase is AQ**G**VN**A** and in YpkA kinase is **A**E**G**ES**H** (*28*). A potential glycine loop in CIII, therefore, is **F**T**G**SN**G**. The S is frequently found after the second G in other kinases, including PKA-Cα (*28*). In PKA-Cα, the glycine loop and K72 are separated by 16 residues, while in cdc7 there are 30 intervening amino acids (*28*). Copines also have an invariant lysine, but 36 residues separate it from the putative glycine loop. This K is conserved in all human copines and *Paramecium tetraurelia*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* homologues (*17*). Most kinases have an alanine two residues prior to the conserved lysine, and CIII does as well. CI, CVI ,and CVII, on the other hand, have a serine in that position. Subdomain III in the classic kinase domain has a glutamic acid (E91 in PKA-Cα) 19 residues downstream of the invariant lysine (K72). CI and CIII also have a glutamic acid at that distance from their invariant lysine. CVI and CVII have an aspartic acid instead, as does the kinase TGFβRII (*28*).

The D166 located in subdomain VIB and D184 in subdomain VII of PKA-Cα are also found at the expected intervals in all four copines. However, residues corresponding to N171 and G186 of PKA-Cα, and conserved in all other kinases, were absent from copines. Up to and including subdomain VII, copines do have some homology with the traditional kinases, with noted exceptions, but no similarities were detected when subdomains VIII−XI were compared with the last 80 amino acids of copines. The expected secondary structure of human CI (based on the algorithm of Chou and Fasman) (*17*) does not seem to correlate with the secondary structure of PKA-Cα determined by crystallography. Function and importance of the secondary structure of copines remain speculative until the crystal structure is available.

The primary sequence of the carboxyl terminus of copines shows homology to the A domain of integrins. The A domain adopts a secondary α∠β∠α structure resembling the classical "Rossmann fold" or dinucleotide binding pocket. The Rossmann fold is found in intracellular enzymes, such as GAPDH and NAD^+ dehydrogenases, that can bind nicotinamide adenine dinucleotide (NAD) (*29*, *30*). The carboxyl terminus of CIII has higher homology with the carboxyl termini of CI, CVI, and CVII when compared to overall homology (69% vs 63%, 60% vs 52%, and 63% vs 47%, respectively) and may have a critical function, such as phosphotransferase activity. Rabbit muscle CI was shown to bind an ATP affinity column in a calcium-dependent fashion (*17*). The combination of the biochemical data, the primary protein sequence, and the predicted secondary structure of the copine C terminus adopting a nucleotide binding motif structure makes it plausible to propose that copines represent a novel kinase family, lacking the complete traditional kinase catalytic domain.

Further biochemical and molecular characterization and determination of interacting proteins are required before any biological role for CIII can be determined. Our data suggest that CIII is a phosphoprotein that has intrinsic kinase activity. Other copine family members may have kinase activity as well. Calcium is likely to play an important role in the putative membrane localization of CIII, and possibly, in its enzymatic function. The importance of phosphorylation of serine and threonine residues in CIII requires further study. In conclusion, this study describes, for the first time, the phosphorylation and associated kinase activity of the widely expressed protein CIII.

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