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Interaction between the catalytic and modifier subunits of glutamate-cysteine ligase

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ABSTRACT

Glutamate-cysteine ligase (GCL) is the rate-limiting enzyme in the glutathione (GSH) biosynthesis pathway. This enzyme is a heterodimer, comprising a catalytic subunit (GCLC) and a regulatory subunit (GCLM). Although GCLC alone can catalyze the formation of L-γ-glutamyl-L-cysteine, its binding with GCLM enhances the enzyme activity by lowering the K_m for glutamate and ATP, and increasing the K_i for GSH inhibition. To characterize the enzyme structure–function relationship, we investigated the heterodimer formation between GCLC and GCLM, in vivo using the yeast two-hybrid system, and in vitro using affinity chromatography. A strong and specific interaction between GCLC and GCLM was observed in both systems. Deletion analysis indicated that most regions, except a portion of the C-terminal region of GCLC and a portion of the N-terminal region of GCLM, are required for the interaction to occur. Point mutations of selected amino acids were also tested for the binding activity. The GCLC Cys248Ala/Cys249Ala and Pro158Leu mutations enzyme showed the same strength of binding to GCLM as did wild-type GCLC, yet the catalytic activity was dramatically decreased. The results suggest that the heterodimer formation may not be dependent on primary amino-acid sequence but, instead, involves a complex formation of the tertiary structure of both proteins.

1. Introduction

Glutathione (GSH) is the most abundant small-molecular-weight thiol in mammalian cells. GSH serves diverse cellular functions [1–3] including: (a) an antioxidant and, with glutathione disulfide, an important cellular redox couple; (b) a substrate for antioxidant and xenobiotic protective enzymes [4–6]; (c) a modifier of proteins post-translationally [7]; (d) a cofactor that reduces ribonucleotides and several lipid second-messengers; (e) a neurotransmitter. GSH is a tripeptide and is synthesized by the sequential actions of glutamate-cysteine ligase (GCL) and glutathione synthetase (GSS). GCL

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Abbreviations: GCL, glutamate-cysteine ligase; *Gclc* and GCLC, the mouse gene and cDNA/mRNA/enzyme, respectively, for the catalytic subunit of GCL; *GCLC* and GCLC, the human gene and cDNA/mRNA/enzyme, respectively, for the catalytic subunit of GCL; *Gclm* and GCLM, the mouse gene and cDNA/mRNA/enzyme, respectively, for the modifier subunit of GCL; *GCLM* and GCLM, the human gene and cDNA/mRNA/enzyme, respectively, for the modifier subunit of GCL; GSH, reduced glutathione; GSSG, oxidized dimer of glutathione; SV40, simian virus-40; AD, activation domain; BD, DNA-binding domain

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catalyzes the rate-limiting step of GSH biosynthesis, using glutamate and cysteine in an ATP-dependent reaction to produce γ-glutamylcysteine (γ-GC). In higher eukaryotes, GCL in its most catalytically active form is a heterodimer, comprised of a catalytic subunit (GCLC) and a modifier subunit (GCLM); these are encoded by evolutionarily unrelated genes on different chromosomes [8].

Studies with both native and recombinant GCL proteins have shown that GCLC, but not GCLM, is active in γ-GC synthesis [9,10]. Furthermore, mice with targeted disruption of the *Gclc* gene demonstrate the essential role for this gene (and, most likely, GSH) for embryonic development [11,12]. On the other hand, the catalytic character of the GCL holoenzyme differs dramatically from GCLC alone. Therefore, GCLM is thought to function by interacting with GCLC and increasing the efficiency of γ-GC synthesis. Thus, GCLM interacts with GCLC to decrease the K_m for glutamate and ATP, increase the K_m for feedback inhibition by GSH, and increase the velocity of γ-GC biosynthesis [10,13–16]. In accord with this function, targeted disruption of the *Gclm* gene leads to dramatic decreases in cellular levels of GSH [16]. Recent studies agree that, in most tissue types studied, GCLC is present in several-fold excess of GCLM [10,17]. These studies underscore the importance of the GCLC/GCLM heterodimer in maintaining GSH levels and further suggest that GSH levels might be controlled by way of GCLM synthesis.

Patients having rare polymorphisms in either the *GCLC* or *GSS* gene have convinced us about how low GSH levels can lead to certain disorders; such patients present complex symptomatology—often including neurological disorders and hemolytic anemia [18–20]. More recently, common polymorphisms in either the *GCLC* or *GCLM* gene, which more modestly impact GSH synthesis, were reported to be independent risk factors for myocardial infarction [21–23]. GSH is also essential in the defense of the central nervous system against oxidative stress, and disturbed GSH homeostasis has been implicated in several neurodegenerative disorders [24]. In a recent report, case–control studies demonstrated significantly decreased GCLM mRNA levels in fibroblasts from schizophrenic patients, and a strong association of schizophrenia with the *GCLM* gene [25]. As a paradox with its beneficial roles, up-regulation of GSH synthetic capacity is found in many tumor types [26–28]; these increases often occur in conjunction with increased GSH transport by multidrug resistance-related proteins [29,30] and resistance to chemotherapy. Such studies underscore the need to understand better the mechanisms that control GSH biosynthesis.

Despite demonstrations both in vitro and in vivo that the GCLC/GCLM holoenzyme is in large part responsible for determining GSH levels, the primary structure of the subunits that determine interaction have not been elucidated. In some studies dissociation of GCLC and GCLM requires reducing reagent, indicating covalent interactions between the cysteine residues of the subunits [13,31]. The importance of disulfide formation to the association of GCLC and GCLM has not been evaluated.

Baker's yeast, *Saccharomyces cerevisiae*, is a genetic model organism that holds promise for elucidating the structural determinants that allow GCLC/GCLM interaction. This is a consequence of a sensitive assay system that can measure quantitatively the interaction of two proteins, i.e. the yeast two-hybrid system. If interaction between the GCLC and GCLM proteins occur in yeast, this model system would also be amenable to screening mutants of GCLC or GCLM in human populations. Because null mutants for all genes, including the yeast "*GCL* gene" (termed *GSH1*), are available for experimentation, the yeast system also is attractive for studies that can examine the control of cellular GSH levels. Finally, the elucidation of GCLC- or GCLM-derived peptides that maintain interaction while losing catalytic activity should provide a basis for the generation of dominant-negative peptides, which may provide useful reagents both for research and perhaps cancer therapy. In this report, we examine the interaction of the primary structural determinants of the mouse GCLC and GCLM protein interaction, in vitro using affinity chromatography, and in vivo using the yeast two-hybrid system.

2. Materials and methods

2.1. Bacterial and yeast systems

All bacterial plasmids, except for histidine-tagged expression vectors, were maintained in *E. coli* DH5α (Stratagene). The expression vectors were transformed in *E. coli* BL21(DE3)pLysS (Invitrogen). *S. cerevisiae* strain HF7c (Clontech) was used for the yeast two-hybrid analysis.

2.2. Plasmid constructs

The full-length mouse GCLC and GCLM cDNAs were obtained by PCR amplification from C57BL/6J mouse liver total RNA. PCR products were cloned into the appropriate vectors, using restriction enzymes and sites added to PCR primers. The sequence of both the GCLC and the GCLM cDNA was verified. Mutant GCLC or GCLM expression vectors were generated in Bluescript II KS(+/−) by PCR alone, or by PCR using the Quickchange II (Stratagene) primers listed in Table 1. The pBluescript II KS(+/−) (Stratagene) (pBS) was used in the TNT-T3 Coupled Wheat Germ Extract System (Promega). The pRSET vectors (Invitrogen) were used to make histidine-tagged proteins. The pAS2-1 and pACT2 vectors (Clontech) were used for the yeast two-hybrid system. All constructs were verified by sequencing.

2.3. Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed using the Matchmaker system (Clontech). Briefly, yeast strain HF7c was first transformed by electroporation with plasmid pAS2-1, containing GCLM (or GCLC). A single clone was further transformed with plasmid pACT2, containing GCLC (or GCLM). The double transformants were then plated on yeast minimal medium (1 M sorbitol, 1.7 g yeast nitrogen base/l, 1 g ammonium sulfate/l, 10 g/l succinic acid/l, 6 g NaOH/l, 20 g dextrose/l, 2% (w/v) agar, and a mixture of essential amino acids minus leucine and tryptophan). For β-galactosidase assays, minimal medium minus leucine and tryptophan was inoculated with a single yeast colony and grown overnight at 30 °C to an OD_{600} of 0.8. Cells were harvested by centrifugation at 3000 × *g* for

Table 1 – Plasmids constructs used in the present study

Construct	Description
pBS-GCLC	GCLC PCR fragment inserted into *Bam*H1 and *Not*I sites in pBS
pBS-GCLM	GCLM PCR fragment inserted into *Bam*H1 and *Not*I sites in pBS
pACT2-GCLC	GCLC PCR fragment inserted into *Bam*H1 and *Sac*I sites in pACT2
pACT2-GCLM	pBS-GCLM digested with *Sma*I and *Sac*I, fragment ligated into pACT2
pAS2-1-GCLC	GCLC PCR fragment inserted into *Eco*R1 and *Bam*H1 sites in pAS-2-1
pAS2-1-GCLM	GCLM PCR fragment inserted into *Bam*H1 site in pAS2-1
pACT2-GCLCΔ1–41	GCLC Δ1–41 PCR fragment inserted into *Bam*H1 and *Eco*R1 sites in pACT2
pACT2-GCLCΔ1–83	GCLC Δ1–83 PCR fragment inserted into *Bam*H1 and *Eco*R1 sites in pACT2
pACT2-GCLCΔ1–175	GCLC Δ1–175 PCR fragment inserted into *Bam*H1 and *Eco*R1 sites in pACT2
pACT2-GCLCΔ1–241	GCLC Δ1–241 PCR fragment inserted into *Bam*H1 and *Eco*R1 sites in pACT2
pACT2-GCLCΔ491–637	pACT2–GCLC mutated to stop codon at GCLC-491
pACT2-GCLCΔ431–637	pACT2–GCLC mutated to stop codon at GCLC-431
pACT2-GCLCΔ256–281	pBS-GCLC 281–256 PCR fragment digested with *Sfo*I and re-ligated, then digested with *Bam*H1 and *Eco*R1, fragment ligated into pACT2
pACT2-GCLCΔ281–340	pBS-GCLC 340–281 PCR fragment digested with *Sfo*I and re-ligated, then digested with *Bam*H1 and *Eco*R1, fragment ligated into pACT2
pACT2-GCLCΔ346–417	pBS-GCLC 417–346 PCR fragment digested with *Sfo*I and re-ligated, then digested with *Bam*H1 and *Eco*R1, fragment ligated into pACT2
pACT2-GCLCP158L	pACT2-GCLC mutated to Pro at GCLC-158
pACT2-GCLCC248AC249A	pACT2-GCLC mutated to Ala at GCLC-248,249
pACT2-GCLMΔ1–42	GCLMΔ1–42 PCR fragment inserted into *Sma*I and *Sac*I sites in pACT2
pACT2-GCLMΔ1–65	GCLMΔ1–65 PCR fragment inserted into *Sma*I and *Sac*I sites in pACT2
pACT2-GCLMΔ1–114	GCLMΔ1–114 PCR fragment inserted into *Sma*I and *Sac*I sites in pACT2
pACT2-GCLMΔ218–274	GCLMΔ218–274 PCR fragment inserted into *Sma*I and *Sac*I sites in pACT2
pACT2-GCLMΔ112–274	GCLMΔ112–274 PCR fragment inserted into *Sma*I and *Sac*I sites in pACT2
pRSETA-GCLC	pBS-GCLC digested with *Bam*H1 and *Not*1, fragment ligated into *Bam*H1 and *Pvu*II sites in pRSETA
pRSETB-GCLM	pBS-GCLM digested with *Bam*H1 and *Not*I, fragment ligated into *Bam*H1 and *Pvu*II sites in pRSETB
pRSETC-GCLCP158L	pACT2-GCLCP158L digested with *Bam*H1 and *Xho*I, fragment ligated into pRSETC
pRSETC-GCLCC248AC249A	pACT2-GCLCC248AC249A digested with *Bam*H1 and *Xho*I, fragment ligated into pRSETC
pRSETC-GCLCΔ1–41	pACT2-GCLCΔ1–41 digested with *Bam*H1 and *Xho*I, fragment ligated into pRSETC
pRSETC-GCLCΔ346–417	pACT2-GCLCΔ346–417 digested with *Bam*H1 and *Xho*I, fragment ligated into pRSETC

10 min at 4 °C and were resuspended in 300 μl of ice-cold Z buffer (0.06 M $Na_2HPO_4{\cdot}7H_2O$, 0.04 M $NaH_2PO_4{\cdot}H_2O$, 0.01 M KCl, 1 mM $MgSO_4{\cdot}7H_2O$; pH 7.0). Cells were then lysed by two cycles of freezing in liquid nitrogen and thawing in 37 °C of water. Crude extract (100 μl) was added to 800 μl of buffer Z, containing the substrate *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml). The reaction was incubated at 30 °C and stopped at various time points by adding 400 μl of 1 M Na_2CO_3. β-Galactosidase activity was assayed by measuring absorbance at 405 nm and normalized against relative numbers of cells. In all cases, the amount of yeast-fusion proteins, whether full-length or mutants, was determined by monoclonal antibodies to either the transactivation or DNA-binding domain of the GAL4 protein (Clontech).

2.4. *In vitro transcription/translation*

Plasmid pBS-GCLM or pBS-GCLC was used in an in vitro transcription/translation reaction with the TNT-T3 Coupled Wheat Germ Extract System (Promega). The full-length protein was radiolabeled with [^{35}S]methionine (specific radioactivity > 1000 Ci/mmol, 10 mCi/ml; NEN), according to the manufacturer's protocol. Translated proteins were pre-cleared through Ni-NTA agarose (Qiagen) and one-tenth equivalent of a translation reaction was used for each interaction study. Labeled proteins were kept at −20 °C until used.

2.5. *Purification of histidine-tagged protein*

E. coli BL21(DE3)pLysS was transformed with plasmid pRSET-GCLC or pRSET-GCLM, using the $CaCl_2$ method (Invitrogen). Bacterial cultures (500 ml) were grown to an OD_{600} of 0.5–0.6, after which the synthesis of fusion protein was induced by the addition of isopropyl thio-β-D-galactoside (IPTG) (1 mM final concentration). After 3 h, the bacteria were harvested by centrifugation and the pellet stored at −70 °C until use. The frozen bacterial cells were lysed by sonication in 30 ml of buffer A [20 mM Tris/HCl, 500 mM KCl, 10 mM imidazole, 10% (v/v) glycerol, 1 mM phenylmethylsulphonyl fluoride, 1% (v/v) Nonidet P40; pH 7.9]. After centrifugation, the supernatant was loaded onto a Ni-NTA column (0.5-ml bed volume) that had been pre-equilibrated with buffer B [20 mM Tris/HCl, 500 mM KCl, 10 mM imidazole, 10% (v/v) glycerol; pH 7.9]. The column was then washed with 10 ml of buffer B, followed by 5 ml of buffer C [20 mM Tris/HCl, 150 mM KCl, 30 mM imidazole, 10%

(v/v) glycerol; pH 7.9]. Bound protein was eluted from the column by 1 ml of buffer D [20 mM Tris/HCl, 150 mM KCl, 300 mM imidazole, 10% (v/v) glycerol; pH 7.9] and immediately dialyzed against either buffer E [10 mM Tris/HCl, 1 mM EDTA, 5 mM $MgCl_2$; pH 8.4] for the purpose of activity assay, or buffer F [50 mM Tris/HCl, 5 mM $MgCl_2$, 5 mM L-glutamate; pH 7.4] for the binding assay. Proteins were quantified using the BCA protein assay kit (Pierce) and stored at −20 °C after addition of glycerol to a final concentration of 25%.

2.6. *In vitro binding assay*

Purified histidine-tagged GCLC or GCLC mutant protein (10 μg) was bound to Ni-NTA agarose (0.030 ml bed volume), and the column was washed three times with buffer G [20 mM Tris/HCl, 150 mM KCl, 20 mM imidazole; pH 7.4]. As a control, lysate (10 μg total protein) from *E. coli* transformed with the empty pRSET vector was used. The protein-bound agarose was then incubated with an aliquot of radiolabeled GCLM in buffer G at 4 °C for 4 h on a rocking platform. As indicated, incubation reactions sometimes contained other compounds to assess their effect on subunit interaction. After washing four times with buffer H [20 mM Tris/HCl, 150 mM KCl, 20 mM imidazole; pH 7.4], bound proteins were eluted with 0.1 ml buffer I [20 mM Tris/HCl, 150 mM KCl, 300 mM imidazole; pH 7.4]. All samples were denatured in an equal volume of Laemmli buffer and separated by SDS-PAGE. [^{35}S]methionine-labeled proteins were detected by autoradiography and quantified with a PhosphorImager (Molecular Dynamics).

2.7. *Western immunoblot analysis*

For the detection of yeast-fusion proteins, whether wild-type or mutants, Western immunoblots were conducted, using mouse monoclonal antibodies to either the activation domain or the DNA-binding domain of the GAL4 protein (1:1000 dilution; Clontech). For the detection of histidine-tagged protens, whether wild-type or mutants, Western immunoblots were conducted using mouse monoclonal anti-HisG antibody (1:5000 dilution; Invitrogen).

2.8. *GCLC activity assay*

GCLC activity was determined by measuring directly the rate of γ-GC formation using HPLC. The reaction mixtures (200 μl) contained buffer J [100 mM Tris/HCl, 50 mM KCl, 15 mM ATP, 20 mM $MgCl_2$, 1 mM Na_2EDTA, 3 mM dithionite, 15 mM sodium L-glutamate; pH 7.8] and purified GCLC or mutant GCLC protein (1 μg). Reactions were allowed to proceed for 30 min at 37 °C, upon adding 3 mM L-cysteine and then stopped by addition of trichloracetic acid; samples were immediately derivatized and analyzed by HPLC [16]. Activities were reported as nmol γ-GC formed per min per mg protein.

2.9. *Statistics*

Statistics were performed using SigmaStat Statistical Analysis software (SPSS Inc., Chicago, IL) or SAS (Statistical Analysis System, Cary, NC). All data are reported as the means ± S.D. Group means were compared by one-way ANOVA, followed by Student's *t*-test for pairwise comparison of means. All p values of <0.05 were regarded as statistically significant.

3. Results

3.1. *GCLC and GCLM interact in yeast and in vitro*

The full-length GCLC and GCLM cDNAs were cloned into the yeast two-hybrid vectors, resulting in the NH_2-terminal in-frame fusion of either the GAL4 DNA-binding domain (BD) or the GAL4 activation domain (AD). The plasmids were then transformed in *S. cerevisiae* HF7c and analyzed for the induction of β-galactosidase activity (Fig. 1). Co-expression of the empty vectors, together or with either GCLC or GCLM, resulted in no detectable β-galactosidase activity above background, whereas co-expression of GCLC and GCLM led to β-galactosidase levels comparable to the company's positive control pairs, pVA3-1 and pTD1-1, which measure interaction between p53 and SV40 large T antigen. Signal-to-noise for interaction between GCLC and GCLM was >100-fold. As a further demonstration of specificity, we found that neither GCLC nor GCLM interacts with itself. Furthermore, neither GCLC nor GCLM interacted with SV40 large T antigen or p53 (data not shown).

The results from yeast were supported in an in vitro binding assay. GCLC was tagged with six histidines at the NH_2-terminus and purified to near homogeneity by way of a Ni-NTA column. GCLM was translated in vitro in the presence of [^{35}S]methionine. The purified GCLC protein was first immobilized to Ni-NTA agarose, followed by the addition of [^{35}S]GCLM. Samples eluted by imidazole were analyzed by electrophoresis. In all affinity-chromatography experiments, gel electrophoresis was used to monitor [^{35}S]GCLM in the original lysate, wash, and eluate. GCLC was also detected in the eluate by Western immunoblot to its histidine tag, to assess its binding to the Ni column. To conserve space in figures subsequent to Fig. 2A, only eluates are shown.

Control columns did not retain any detectable amount of labeled GCLM, whereas a significant fraction of the input [^{35}S]GCLM was bound to the GCLC-attached agarose (Fig. 2A). In vitro translation of GCLM resulted in the synthesis of

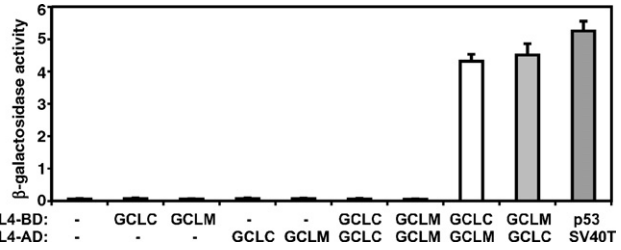

Fig. 1 – Interaction of the GCLC and GCLM proteins in yeast. Yeast-fusion proteins with GAL4 DNA binding domain (BD) and GAL4 activation domain (AD), as indicated, were co-expressed in yeast strain HF7c; strength of interaction was measured as β-galactosidase activity. Binding between p53 and SV40 large T antigen (SV40T) was used as the positive control. Data are reported as means ± S.D. of three independent transformants.

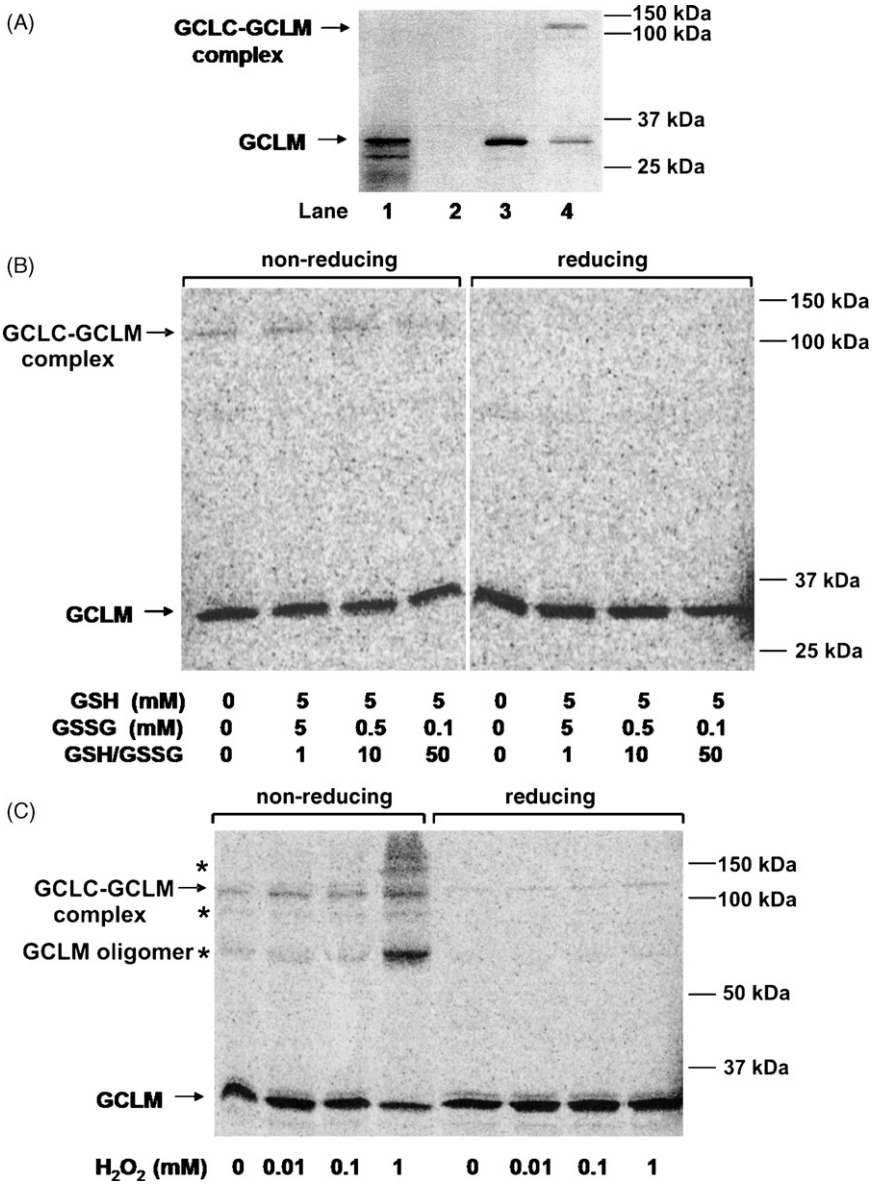

Fig. 2 – Interaction of GCLC and GCLM on Ni-NTA resin. [^{35}S]GCLM was added to Ni-NTA resin, preincubated with either control *E. coli* lysate or histidine-tagged GCLC. The resin was then washed and eluted with imidazole. (A) Eluates (30 μl) from each fraction were subjected to SDS-PAGE under reducing conditions (5% β-mercaptoethanol) except for lane 4. Lane 1, wheat germ lysate containing [^{35}S]GCLM; lane 2, eluate from control *E. coli* lysate-bound resin; lane 3, eluate from histidine-tagged GCLC-bound resin; lane 4, eluate from histidine-tagged GCLC-bound resin without reducing agent. (B) Effects of GSH plus GSSG, on the GCLC–GCLM interaction. Binding of [^{35}S]GCLM to histidine-tagged GCLC on Ni-NTA resin was performed in the presence of the indicated concentrations of GSH and GSSG. Eluates (30 μl) were loaded onto SDS-PAGE under non-reducing conditions, or reducing conditions, as indicated. (C) The interaction of GCLC with GCLM was allowed to proceed in binding buffer containing the indicated concentrations of H_2O_2. Eluates (30 μl) were loaded on SDS-PAGE under non-reducing conditions, or reducing conditions, as indicated.

full-length GCLM and some smaller products that were likely the result of translational initiation at internal methionine codons. From this mixture, only full-length GCLM was found to interact with GCLC. Both GCLM and the heterodimeric form were present on the non-reducing SDS-PAGE gel; however, under reducing conditions, only GCLM was seen on the gel suggesting that the subunits interaction involves both non-covalent and disulfide bonds. The converse experiment, in which GCLM was tagged with histidine and GCLC was radiolabeled, gave similar results under reducing conditions (data not shown).

The results in Fig. 2A represent initial experiments conducted to test the assay system. In subsequent experiments, GCLM was found to bind to GCLC with similar fidelity, but the appearance of the GCLC–GCLM heterodimer was variable, usually constituting a minuscule portion of the detected [^{35}S]GCLM. To further investigate factors that might promote the disulfide formation between the subunits, we

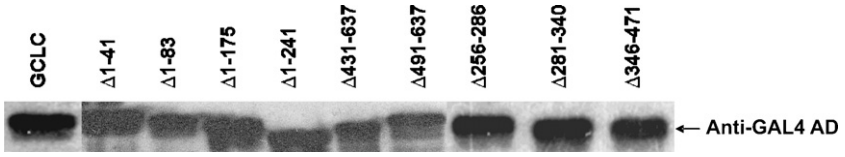

Fig. 3 – Immunodetection of GCLC fusion proteins in yeast lysates. Yeast strain HF7c was transformed with the pACT2 plasmid containing GCLC or its deletions. Yeast lysates (20 μg) were loaded for SDS-PAGE. The amount of GCLC, or its truncation mutants, was detected by Western immunoblot analysis using mouse monoclonal anti-GAL4-AD (1:1000 dilution).

conducted interaction incubations with various ratios of the GSH/GSSG redox couple. GSH/GSSG (50:1) represents the physiological ratio of the redox couple in resting cells. GSH/GSSG (1:1) represents the oxidized couple at a supraphysiological level. The level of GSH (5 mM) used in these experiments is at the high end found in cells. Even at this concentration, GSH did not affect the interaction of GCLC with GCLM, and decreasing the GSH/GSSG ratio did not increase heterodimer formation (Fig. 2B). Reactions were also carried out, using each of the substrates (cysteine, ATP and glutamate), and no change in the GCLC–GCLM interaction or heterodimer formation was observed (data not shown).

We also assessed whether the strong physiological oxidant H_2O_2 would promote heterodimer formation. As depicted in Fig. 2C, H_2O_2 did not promote heterodimer formation even at concentrations that caused oligomerization of GCLM. As previously noted, oligomers of GCLM have been detected in stained gels, when GCLM is incubated at high concentrations in the absence of reductant. A similar observation has been reported for *Drosophila* recombinant GCLM [32]. The physiological significance of this observation, if any, is not known.

We conclude that the formation of covalent interactions between GCLC and GCLM are not obligatory to interaction and, when observed, may represent only an artifact of in vitro interaction.

Taken together, interaction of GCLC with GCLM in yeast two-hybrid assays, or in vitro, gave qualitatively similar results.

3.2. *Multiple interacting regions exist in GCLC and GCLM*

In order to delineate regions within the GCLC and GCLM proteins that are responsible for their interaction, we designed a series of NH_2- and COOH-terminal deletions, as well as several internal deletions based on the predicted protein secondary structure. These deletion constructs were fused to the GAL4 AD domain, and the strength of interaction with the corresponding partner was measured as β-galactosidase activity in the yeast two-hybrid system. All of the fusion proteins were expressed at similar levels, as revealed by Western immunoblot analysis using antibodies to either the GAL4-BD or GAL4-AD suggesting that fusion proteins are stable in yeast. Fig. 3 shows a representative Western immunoblot of GCLC–GAL4 AD, and similarly designed GCLC mutant fusion proteins.

The data in Fig. 4 summarize the results from the nine GCLC deletions. Deletion of the first 41 residues at the NH_2-terminal end was sufficient to completely abolish binding to GCLM. The COOH-terminal end appeared to be less critical, since the construct containing a deletion of the last 146 residues retained 29% of full binding activity. However, expanding this deletion up to residue 431 (Δ431–637) resulted in complete loss of binding indicating the location of a segment important for binding between residues 431 and 491. An internal deletion of amino acid-346 through −417 (Δ346–417) gave a modest interaction with GCLM (23% of the full-length protein), whereas constructs Δ256–281 and Δ281–340 showed no binding activity.

In GCLM, the deletion of the last 56 COOH-terminal residues caused complete loss of binding activity (Fig. 5), whereas deletions that removed the NH_2-terminal first 42 (mutant Δ1–42) or 65 (mutant Δ1–65) residues gave a dramatic reduction in binding strength (35% and 15% of that seen with the full-length protein, respectively). The construct with residues 1–114 deleted (Δ1–114) showed almost no binding activity. In summary, the NH_2-terminal region in GCLC and the COOH-terminal region in GCLM both appear to be required for subunit binding; however, internal segments of both proteins are also critical for their interaction.

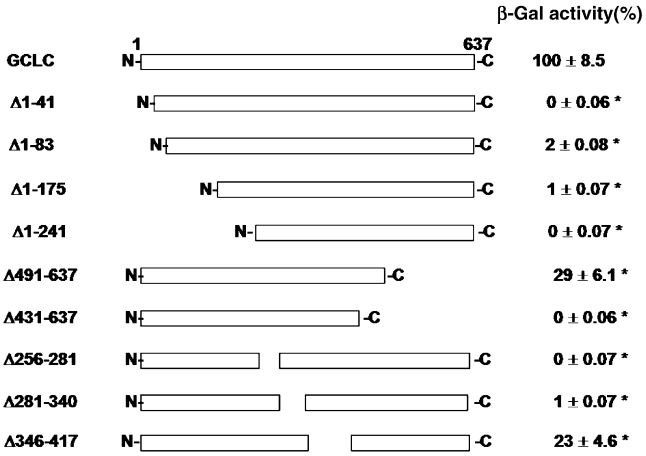

Fig. 4 – GCLC deletion studies in the yeast two-hybrid system. Yeast strain HF7c was sequentially transformed with the pAS2-1 plasmid containing GCLM, and with the pACT2 plasmid containing GCLC, or GCLC carrying truncations. The strength of interaction between GCLC and GCLM in each transformant was determined by the percentage of β-galactosidase activity, relative to that determined using the full-length GCLC. Data are expressed as means ± S.D. of three independent transformants. Asterisk (*) denotes $p < 0.001$.

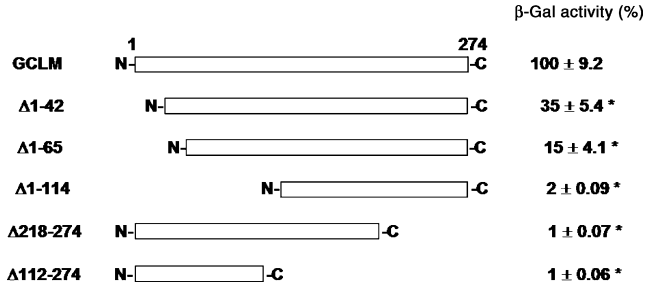

Fig. 5 – GCLM deletion studies in the yeast two-hybrid system. Yeast strain HF7c was sequentially transformed with the pAS2-1 plasmid containing GCLC, and with the pACT2 plasmid containing GCLM, or GCLM carrying truncations. The strength of interaction between GCLC and GCLM in each transformant was determined by the percentage of β-galactosidase activity, relative to that determined using the full-length GCLM. Data are expressed as means ± S.D. of three independent transformants. Asterisk (*) denotes $p < 0.001$.

Several of the truncated GCLC and GCLM proteins were tested in vitro for interaction. Results mimicked the results seen in vivo. In general, interactions in yeast that were >20% of wild type were detected as less intense bands by affinity chromatography, but weaker interactions were not detected in vitro (representative interactions shown in Fig. 6C).

3.3. *The catalytic activity of GCLC is partly dissociable from its binding with GCLM*

Because GCLC contains the ability to bind with GCLM, as well as the catalytic activity of γ-GC formation, we asked whether the two properties could be dissociable. A GCLC protein that was devoid of catalytic activity but still competent to bind GCLM should exert a dominant-negative effect, when over-expressed by binding GCLM. Protein sequence alignment shows that the two consecutive cysteines (C248, C249) are conserved in most taxa and suggests that these two cysteines are essential for catalysis [33]. Mutations in either of these cysteines decreased the enzymatic activity of human GCLC to 10–15% [34]. Perhaps mutating both cysteines would eliminate catalysis by GCLC. In addition, a mutant Pro158Leu in the GCLC protein having low catalytic activity has been described in humans with GSH deficiency. Patients homozygous for this mutation have only 2% of normal GSH levels in erythrocytes [20]. Based on this information, we made one GCLC construct with the two point mutations, Cys248Ala and Cys249Ala, and another construct with the mutation Pro158Leu; these were inserted into the histidine-tagged expression vector, and the enzymatic activities of the resulting proteins were then determined (Fig. 6A) and compared with the activity of wild-type GCLC.

Both mutant proteins had greatly decreased GCLC catalytic activity. GCLC Pro158Leu retained only 7.5% of wild-type GCLC catalytic activity. Next, we determined the interaction of these proteins, both in vivo using the yeast two-hybrid assay (Fig. 6B)

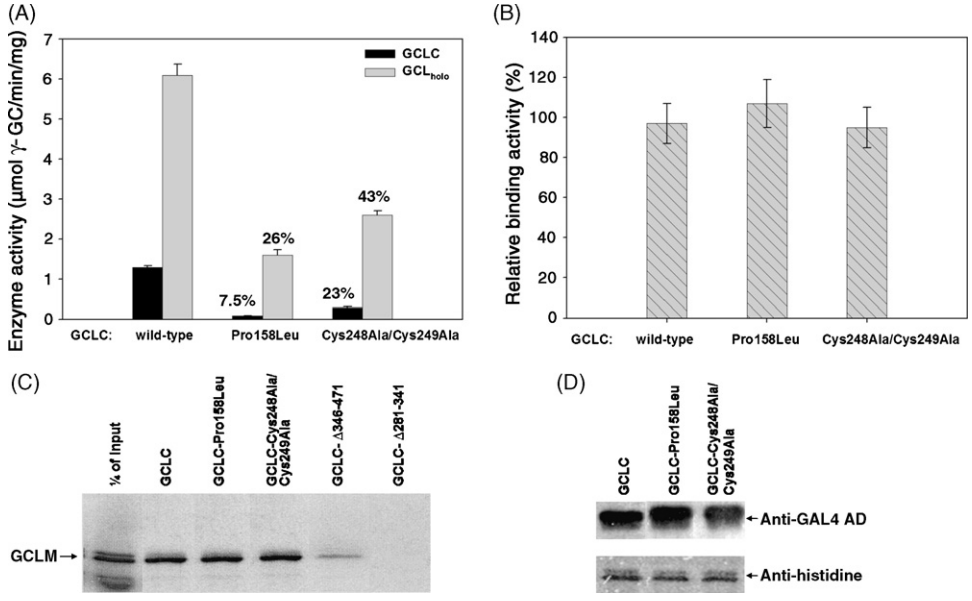

Fig. 6 – Catalytic activity of wild-type GCLC versus mutant GCLC proteins, compared with binding to GCLM. (A) Enzyme activity of GCLC was analyzed by measuring γ-GC production without GCLM (*i.e.* GCLC assayed alone) or with a 2-fold molar excess of GCLM (*i.e.* GCL_{holo}). Data are reported as means ± S.D. of three independent experiments. (B) The interaction of GCLC mutants with GCLM was tested in the yeast two-hybrid system. GCLC and its mutants were fused to the GAL4 activation domain and GCLM to the GAL4 DNA-binding domain. Strength of interaction was measured as β-galactosidase activity, and results are expressed as percent of the interaction observed between wild-type GCLC and GCLM. (C) Interaction of [^{35}S]GCLM with wild-type GCLC versus mutant GCLC proteins on Ni-NTA resin. The conditions of interaction are described in Fig. 2 legend. Extracts were analyzed by SDS-PAGE under reducing conditions. (D) Western immunoblot showing the accumulation of GCLC and its mutants in yeast lysates (20 μg, top gel) using mouse monoclonal anti-GAL4-AD (1:1000), and in *E. coli* lysates (1 μg, bottom gel) using mouse monoclonal anti-HisG (1:5000).

described above, and in vitro using [^{35}S]-labeled GCLM synthesized in wheat germ extract (Fig. 6C). In both assays, the mutant GCLC proteins interacted with GCLM similarly to that of wild-type GCLC. We next assessed the ability of GCLM to alter the catalytic activity of the mutant GCLC proteins. These assays were conducted by complementing GCLC wild-type or mutant protein with a 2-fold molar excess of GCLM, as previously described [10]. Complementing wild-type GCLC with GCLM resulted in a 4.6-fold increase in the generation of γ-GC. Complementation of GCLC Pro158Leu resulted in a 16-fold increase in γ-GC formation, and the holoenzyme containing this mutant had 26% activity of that for the wild-type holoenzyme. Likewise, complementation of GCLC Cys248Ala/Cys249Ala resulted in a 8.7-fold increase in γ-GC synthesis bringing this mutant to 43% of wild-type holoenzyme catalytic activity. Thus, for both GCLC mutants, complementation with GCLM showed a strong positive effect in restoring GCL activity; moreover, GCLC binding activity with GCLM can be divorced from the catalytic activity of γ-GC formation.

4. Discussion

We conducted the experiments reported herein with several independent goals. First, we wished to understand the role of GSH and GSSG in regulating non-covalent GCLC–GCLM interaction versus covalent GCLC–GCLM interaction. Second, we wished to evaluate whether or not yeast provided a suitable intracellular environment to allow the GCLC–GCLM interaction. Third, if interaction occurs in yeast, we wished to determine if the yeast two-hybrid system would allow the assessment of the relative strength of interaction between mutant GCLC and GCLM proteins. Fourth, we wished to delineate the peptide sequences within the GCLC and GCLM proteins that mediate interaction.

Our study showed that GCLC and GCLM can interact both in yeast and in vitro. About 70% of disulfide-linked subunits have been reported in holoenzyme isolated from rat kidney [31]; on the other hand, impaired intermolecular disulfide linkage between *Drosophila* recombinant GCL subunits has little or no impact on the formation of the holoenzyme [32]. In this current study, covalent heterodimer formation represented a sometimes variable, but generally a very minor, proportion of GCLC and GCLM heterodimers. Furthermore, this portion could not be increased by mild [decreased GSH/GSSG ratio, or GSSG alone (not shown)] or strong (H_2O_2) oxidizing agents. We conclude that covalent interactions between the subunits is not obligatory to interaction and, further, that care must be exercised when manipulating GCLC–GCLM heterodimers, because covalent interactions between the subunits can occur. Such interaction may represent an artifact of the isolation conditions, rather than an accurate measurement of the state of the heterodimer in the cell.

It has previously been suggested that GSH may act, in part, to inhibit catalysis by GCL by means of promoting the dissociation between the subunits [31,32]. Herein we show that GSH (5 mM), which represents the high end of cellular GSH concentrations, is ineffective at disrupting the interaction between GCLC and GCLM. We therefore conclude the inhibitory effect of GSH is likely a direct effect on the heterodimer.

Mammalian GCL holoenzyme is made up of two subunits, whereas *E. coli* and *S. cerevisiae* appear to lack the modifier subunit [35]. Yeast may provide a very powerful system to study factors that influence GSH synthesis in cells. We have postulated that GCLC–GCLM interaction studies in yeast might help us to understand the function of GCLC, GCLM and GSH levels in mammalian cells. In this regard, based on comparison of galactosidase levels that accumulate due to the interaction of GCLC with GCLM, or the interaction of SV40 large T antigen with p53 (a known strong interaction), we conclude that our hypothesis is true. The availability of yeast loss-of-function mutants for all yeast genes, including the yeast *GSH1* gene (encoding a homolog of GCLC), should facilitate in this analysis. Such future studies would include dissecting the molecular mechanisms regulating organellar import and export of GSH (mitochondria, nuclei, and the endoplasmic reticulum are all believed to possess independent pools of GSH), as well as GSH export from the cell [36]. In this regard, we have recently demonstrated that mouse GCLC can substitute for yeast GCLC in restoring growth of yeast in minimal medium (manuscript in preparation).

The yeast two-hybrid system is a powerful tool for detecting protein–protein interactions. This system has helped determine the binding motifs in many interacting proteins [37,38]. In this study, we have attempted to delineate the regions of interaction for the GCLC and GCLM proteins. In our study of GCLC, all of the deletion mutants showed substantially diminished binding capacity to GCLM. However, a few discrete regions appeared to be critical for binding, because deletion of these regions decreases the binding of GCLC to GCLM to background levels. This was the case for residues 1–41, 256–281, and 281–340 in the GCLC protein. Whereas the protein with the COOH-terminal 206 residues deleted (Δ491–637) retained 29% binding compared with that of the wild-type GCLC protein, extending that deletion to residues 431–636 caused complete loss of binding activity. The fact that all of the deletion mutants showed less binding activity in this assay was not due to the expression level nor to stability of the protein, because Western blot analysis detected comparable levels of expression in all yeast fusion proteins.

The in vitro binding assay has further verified the results from yeast two-hybrid studies. Therefore, the lack of binding may be attributed to either the removal of segments involved in dimer formation, or improper folding of the truncated proteins. Computer-assisted analysis has suggested that both GCLC and GCLM form compact globular proteins having no discrete domains in either of the subunits [39,40]. Combined with our experimental data, we hypothesize that heterodimerization of GCLC and GCLM is dependent on the tertiary structures formed by the three-dimensional folding of these proteins. Although the use of deletion mutants in a yeast two-hybrid system was of somewhat limited value in determining interacting protein segments in our study, the β-galactosidase activity does reflect the strength of protein–protein interaction. As Fig. 6 demonstrated, the data correlate fairly well with the in vitro binding assay. Low levels of GSH content have been associated with decreased GCL activities in several studies [19,41,42]. Although most researchers have focused on amino

acid changes that are associated with catalytic activity, changes in binding ability could also dramatically affect the efficiency of the enzyme. A study using *Drosophila* recombinant proteins suggests that both covalent and non-covalent interactions between the GCL subunits are important in regulating the enzyme activity [32]; reversible protein phosphorylation, which may also contribute to interaction of these subunits, has been suggested as another mechanism for regulating GCL activity [43]. The yeast two-hybrid system described here will be an efficient approach to screen for the functional changes of amino acids critical for interaction of the subunits.

We had hoped that deletion mutants of GCLC or GCLM, discovered by interaction analysis, would yield proteins not having catalytic function while maintaining wild-type interaction. Such mutants, when expressed in cells, should act as dominant-negative proteins and thereby be useful in experiments to delineate the importance of heterodimer formation during GSH accumulation and cellular protection against oxidant insult. However, it was soon apparent that such an approach was not possible because interaction, for the most part, requires intact GCLC and GCLM proteins. As a consequence, we also evaluated point mutations in the GCLC protein, in hopes of finding a GCLC enzyme which lacked catalytic activity but retained binding. In this effort we were partially successful, and both the Pro158Leu and Cys248Ala/Cys249Ala mutants showed dramatically reduced catalytic activity, while retaining wild-type binding activity. It was somewhat disappointing, however, that – following interaction of GCLC with GCLM – the catalytic activity of heterodimers containing mutant GCLC were dramatically more catalytically active. This fact may explain why GCLC Pro158Leu, when homozygous, is not lethal. Although red cells from patients homozygous for the GCLC Pro158Leu mutation have extremely low levels of GSH, nucleated cells in the tissues of these patients may compensate for this mutation by over-expressing GCLM. Although such a mechanism is purely speculative, we have noted that *GCLM* gene expression is activated when the *GCLC* gene in liver has been ablated [44]. In addition, attempts to use GCLC Pro158Leu or GCLC Cys248Ala/Cys249Ala as dominant-negative proteins in cell culture led to elevated GSH levels (unpublished observations).

In summary, the GCLC and GCLM proteins interact both in vitro and in vivo. Interaction between the subunits, studied by using the yeast two-hybrid system, should be useful in evaluating the interaction of mutant GCL subunits. Yeast may serve as a useful model system for evaluating the function of GCLC and GCLM in controlling GSH biosynthesis and GSH accumulation.

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