GNIP, a Novel Protein That Binds and Activates Glycogenin, the Self-glucosylating Initiator of Glycogen Biosynthesis*

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Glycogenin is a self-glucosylating protein involved in the initiation of glycogen biosynthesis. Self-glucosylation leads to the formation of an oligosaccharide chain, which, when long enough, supports the action of glycogen synthase to elongate it and form a mature glycogen molecule. To identify possible regulators of glycogenin, the yeast two-hybrid strategy was employed. By using rabbit skeletal muscle glycogenin as a bait, cDNAs encoding three different proteins were isolated from the human skeletal muscle cDNA library. Two of the cDNAs encoded glycogenin and glycogen synthase, respectively, proteins known to be interactors. The third cDNA encoded a polypeptide of unknown function and was designated GNIP (glycogenin interacting protein). Northern blot analysis revealed that GNIP mRNA is highly expressed in skeletal muscle. The gene for GNIP generates at least four isoforms by alternative splicing. The largest isoform GNIP1 contains, from NHI- to COOH-terminal, a RING finger, a B box, a putative coiled-coil region, and a B30.2-like motif. The previously identified protein TRIM7 (tripartite motif containing protein 7) is also derived from the GNIP gene and is composed of the RING finger, B box, and coiled-coil regions. The GNIP2 and GNIP3 isoforms consist of the coiled-coil region and B30.2-like domain. Physical interaction between GNIP2 and glycogenin was confirmed by co-immunoprecipitation, and in addition GNIP2 was shown to stimulate glycogenin self-glucosylation 3–4-fold. GNIPs may represent a novel participant in the initiation of glycogen synthesis.

The biosynthesis of glycogen involves two distinguishable stages. The initiation step involves the formation of a glycoprotein primer, by self-glucosylation of glycogenin to form a covalently linked oligosaccharide. Elongation involves the bulk synthesis of glycogen through the reactions catalyzed by glycogen synthase and branching enzyme (for reviews see Refs. 1–3). Humans express two isoforms of glycogenin, one widely distributed (4, 5) and the other, glycogenin-2, predominantly expressed in liver (6). One of the most important properties of glycogenin is its ability to self-glucosylate, using UDP-glucose as a glucose donor (6, 7). In rabbit skeletal muscle glycogenin-1, self-glucosylation results in the formation of a C-1-O-tyrosyl linkage between glucose and Tyr134 (8, 9). Self-glucosylation continues with the formation of α-1,4-glycosidic linkages, until a chain of 8–12 residues has formed. This form of glycogenin serves as a substrate for glycogen synthase (1–3).

Several lines of evidence suggest an important role of protein–protein interactions for the function of glycogenin. First, glycogenin is capable of forming dimers (10–12), resulting in self-glucosylation via an inter-subunit mechanism (12, 13). It was proposed that interaction between subunits of glycogenin is relatively weak (12). Second, there are protein–protein interactions between glycogenin and glycogen synthase. For example, glycogenin co-purified with glycogen synthase from rabbit skeletal muscle in a stoichiometric 1:1 complex, indicating a relatively strong interaction (7). Moreover, two-hybrid analysis revealed the interaction between yeast glycogenin, Glg2p, and yeast glycogen synthase, Gsy2p (14). Third, protein–protein interactions may be involved in the subcellular distribution of glycogen. For example, glycogenin expressed in mammalian cells as a green fluorescent fusion protein was partly co-localized with actin (15). Binding to actin is mediated by the carboxyl-terminal part of glycogenin. Additionally, glycogenin was found in the cytoplasm and the cell nucleus (15–17).

In order to identify other proteins that interact with glycogenin, we used the yeast two-hybrid system with rabbit glycogenin-1 as bait. We identified an as yet undescribed protein, which we named glycogenin-interacting protein (GNIP),1 that activates glycogenin in vitro.

EXPERIMENTAL PROCEDURES

Plasmids—The cDNA for rabbit skeletal muscle glycogenin was generated by cutting pET15b-GN (11) with NdeI, blunting with Klenow fragment, and subsequent digestion with SauI. The cDNA was ligated into pGEBU-C2 (18) that was cut with EcoRI, blunted, and digested with SauI. The resulting plasmid, which contains the glycogenin coding sequence in-frame with the DNA binding domain of Gal4p, was designated pGEBU-GN. The plasmid containing a cDNA fragment for GNIP, GNIPt-h, flanked by EcoRI-NorI-SauI adapter sequences (pGAD-GNIP-h) was isolated from the two-hybrid library and used to create several GNIP-t expression vectors. To construct pET28-GNIP-h, pGAD-GNIP-h was digested with NorI and blunt-ended, and the 1.6-kb fragment encoding GNIP-h was ligated into pET28a (Novagen), which had been previously cut with NheI and blunt-ended. To construct pFLAG-GNIP-h, pGAD-GNIP-h was digested with NorI, and the 1.6-kb fragment was inserted into pFLAG-CMV-2 (Sigma), which was digested with NorI. To construct the vectors expressing GNIP1, the 0.4-kb fragment encoding the 5’ end of GNIP1 was generated by BglII and NorI digestion of DNA from EST clone A11949 (Fig. 1). The middle fragment of GNIP1 was amplified from a human skeletal muscle library by PCR using GNIP1- and library adapter-specific primers, as described below. This PCR product was digested with NorI and EcoRI to generate a 0.5-kb fragment. Both the 0.4- and 0.5-kb fragments were ligated with BglII- and EcoRI-cut pET32a to construct pET32-GNIP-N.

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1 The abbreviations used are: GNIP, glycogenin-interacting protein; RACE, rapid amplification of cDNA ends; UTR, untranslated region; ORF, open reading frame.
The 1.2-kb fragment encoding the 3'-terminal part of GNP1 was obtained by digestion of pGAD-GNIP1-h with EcoRI and SalI. The 1.2-kb fragment was ligated into EcoRI- and SalI-cut pET32-GNIP-N to produce pET32-GNIP1. To construct pGADT7-GNIP1, pET32-GNIP1 was digested with BamHI and XhoI, and the 2.1-kb fragment was inserted into BamHI and XhoI-cut pGADT7 (CLONTECH). To generate GNIP2, the 0.3-kb fragment of GNIP2 encoding amino acid residues from Met1 to Glu10 was carrying an NdeI site at the 5'-end was generated by PCR. In this product was digested with NdeI and EcoRI to produce the 0.2-kb fragment. The 1.2-kb fragment encoding the 3'-terminal part of GNP2 was obtained by digestion of pGAD-GNIP2-h with EcoRI and XhoI. Both the 1.2-kb and the 2.1-kb fragments were ligated with the 0.2-kb fragment using EcoRI to construct pGADT7-GNIP2. To construct pET28-GNIP2, pGADT7-GNIP2 was digested with NdeI and EcoRI, and the 0.2-kb fragment was used to substitute the 0.3-kb NdeI/EcoRI fragment in pET28-GNIP1-h.

**Yeast Two-hybrid Screen**—The yeast strain PJ69-4A (18) was sequenced and transformed with pBDDU-GN with the human skeletal muscle matchmaker cDNA library in pGAD10 plasmid (CLONTECH) using the lithium-acetate method. The transformants were plated on synthetic medium lacking uracil, leucine, and adenine. Colonies were picked from 4 to 12 plates and re-plated on the second generation plates with synthetic medium deficient in uracil, leucine, and histidine. Library plasmids from the second generation colonies were rescued into Escherichia coli by the pBlueLytre method. Plasmid DNA was purified using Plasmid Miniprep Kit (Bio-Rad), and PCR products were identified by automated DNA sequencing.

By using this protocol, the 5'-end of the original two-hybrid clone (GNP1p-h) was extended (PCR 403, Fig. 1). Search of the human EST data base in GenBankTM using the sequence of clone PCR 403 as a query identified one EST clone (GenBankTM accession number AI 492496) with sequence that partially overlaps the sequence of PCR clone 5-61. This clone was obtained from the ATCC collection and contains GNIP3 (GenBankTM accession number AF396655). The original two-hybrid clone, GNIP1p-h, represents the 3'-end fragment of GNIP1 (Fig. 1). The cDNA for GNIP1 was assembled in pET32a and pCMV-Tag-3B vectors using the sequence of clone PCR 403 as a specific primer (SAS14), 10 pmol of adapter primer (AP1, CLONTECH), and 10 pmol of the gene-specific primer (SAS14), 10× reaction buffer containing 15 mM MgCl2 (Roche Molecular Biochemicals), and 1 unit of Expand High Fidelity Taq polymerase (Roche Molecular Biochemicals) in a total volume of 50 μl. Hot start cycling conditions were used with an initial denaturation step at 94 °C for 2 min followed by addition of the enzyme at 85 °C. This was followed by 30 cycles of 94 °C for 30 s and 65 °C for 45 s and a final extension step at 68 °C for 5 min. The last cycle was at 68 °C for 8 min. For secondary amplification, components and conditions were identical except that the CLONTECH and gene-specific primers (SAS12) primers were used; 1 μl of the primary product was used as template and 35 cycles of 94 °C for 15 s, 68 °C for 30 s, and 68 °C for 5 min were performed. Cloning of amplified products was carried out using TOPO TA Cloning kit (Invitrogen). Plasmid DNA was purified using Plasmid Miniprep Kit (Bio-Rad), and PCR products were identified by automated DNA sequencing.

The assay measures the amount of [14C]glycogenin essentially as described previously (12). After centrifugation of the cell homogenate at 10,000 × g for 30 min, the supernatant was collected and dissolved in 0.5 ml of pLipoFAMINE (Invitrogen) and 1× TriFastLock buffer. The supernatants, the pellet fractions, and the immunoprecipitates were separated by SDS-PAGE loading buffer. The pellets were washed three times with phosphate-buffered saline containing 0.1 M NaCl and 0.1% Triton X-100, and proteins were eluted with SDS-PAGE loading buffer. The supernatants, the pellet fractions, and the immunoprecipitates were separated by SDS-PAGE using Western-specific and λTriFast-specific primers. The largest PCR fragments were selected for sequence analysis. Three clones, PCR38D (GNIP2a), PCR38E (GNIP2b), and PCR39H (GNIP2c). The existence of three alternatively spliced mRNA products for GNIP2 was confirmed by sequencing of several clones obtained from human skeletal muscle Marathon-Ready cDNA as a result of 5'-RACE analysis.
reaction mixture were spotted onto P81 chromatography paper which was washed three times with 5% phosphoric acid for 20 min each. The paper was dried and subjected to scintillation counting. In the second method, an aliquot was added to 0.25 volume of 5× SDS-PAGE loading buffer and subjected to SDS-PAGE and autoradiography.

RESULTS

Screening for Glycogenin-1-binding Proteins Using the Two-hybrid System—To search for cDNA clones encoding proteins that interact with glycogenin-1, we fused the coding sequence for rabbit skeletal muscle glycogenin with the Gal4p DNA binding domain and screened a human skeletal muscle library expressed from the pGAD-10 vector. From 3.1 × 10⁶ transformants harboring bait and library cDNA, five clones were isolated representing three different cDNAs as judged by cDNA sequencing. One clone contained a 3.2-kb cDNA for glycogen synthase including 157 bp of the 5′-untranslated region (clone GN-11). Two clones contained a 1.5-kb cDNA for glycogenin-1 with 82 bp (clone GN-6) or 91 bp (clone GN-10) of 5′-untranslated region. The sizes of the isolated cDNAs exceed the lengths of coding regions for glycogen synthase and glycogenin indicating that both polypeptides are expressed in yeast as full-length proteins, with a segment of the fusion protein coded by the 5′-UTR. Two other clones contained identical 1.6-kb fragments encoding a 340-residue polypeptide of an unknown protein (clones GN-1 and GN-7). This protein was designated glycogenin-interacting protein (GNIP), and the entire protein sequence encoded by two-hybrid library plasmid was designated GNIPt-h (see Fig. 1). To confirm the interactions, the plasmids isolated from two-hybrid clones were introduced into yeast that had been pre-transformed with pGBDU-GN. The resulting transformants were able to express both ADE2 and HIS3 genes, which are under control of the Gal4p upstream-activating sequence in PJ69-4A yeast strain (data not shown). Based on quantitative two-hybrid assay for glycogenin interactions. The plasmid expressing rabbit muscle glycogenin (pGBDU-GN) was tested in combination with plasmids isolated from clones GN-1, -6, and -11 or control pGAD-10 plasmid. Plasmids from these clones express GNIP (GNIPt-h, clone GN-1), glycogen synthase (clone GN-11), or glycogenin itself (clone GN-6). The two-hybrid assay was quantitated using β-galactosidase measurements (see “Experimental Procedures”). Values represent an average of two independent experiments.

GNIP, a Glycogenin-binding Protein

FIG. 1. Schematic of GNIP cDNA clones. The different clones are described in the text. The sequence of the cDNA for GNIP1 and sequences identical to GNIP1 are shown as horizontal thick lines. The region of the clone PCR GP9 with unique sequence is shown as horizontal thin line. The numbers indicate the scale and the length of the cDNA for GNIP1 expressed in base pairs. Restriction sites for BglII, NotI, and EcoRI are indicated by arrows. Putative initiating ATG codons are indicated by dashed vertical ticks. The terminating TGA codon is indicated as solid vertical tick.

FIG. 2. Quantitative two-hybrid assay for glycogenin interactions. The plasmid expressing rabbit muscle glycogenin (pGBDU-GN) was tested in combination with plasmids isolated from clones GN-1, -6, and -11 or control pGAD-10 plasmid. Plasmids from these clones express GNIP (GNIPt-h, clone GN-1), glycogen synthase (clone GN-11), or glycogenin itself (clone GN-6). The two-hybrid assay was quantitated using β-galactosidase measurements (see “Experimental Procedures”). Values represent an average of two independent experiments.

CLONE | IDENTITY
---|---
GN-11 | Glycogen synthase
GN-6 | Glycogenin
GN-1 | GNIPt-h
control | 

β-galactosidase activity (units)

|  | 0 | 10 | 20 | 30 | 40 |
|---|---|---|---|---|---|
| | | | | | |

both FLAG-GNIPt-h and glycogenin. With a specific anti-glycogenin-1 antibody, glycogenin was only detected in immunoprecipitates from cells co-transfected with glycogenin and FLAG-GNIPt-h (Fig. 3C). These data demonstrate that the interaction between glycogenin and GNIP occurs in mammalian cells.

GNIP Contains a B30.2-like Domain and a Predicted Coiled-coil Domain—The nucleotide sequence of the 1579-bp GNIPt-h insert predicted an open reading frame (ORF) encompassing nucleotides 1–1021, in-frame with the GAL-4 activation domain of pGAD-10. The ORF was followed by a TGA stop codon at nucleotides 1022–1024. Near the 5′ end, there was a putative ATG start codon at nucleotide 113. The deduced amino acid sequence of the ORF (bp 113–1021) encodes a protein of 303 amino acids with theoretical molecular mass of 34213 Da. GenBank™ searches of the protein data base using the BLASTP algorithm indicated several homologous proteins including butyrophilin, RET finger protein (RFP), pyrin/marenostin (the Mediterranean fever protein), 52-kDa Ro(SSA autoantigen), and several RING finger and zinc finger proteins of unknown function. The homologous region was at the COOH terminus of these proteins and contained a B30.2-like domain (21). Analysis of the GNIPt-h protein sequence with the Simple Modular Research Tool (SMART) (22) indicated that 114 residues of COOH terminus contain a SPRY domain, which is a subdomain of the B30.2 region (23, 24). Toward the NH₂ terminus of GNIPt-h, the SMART program identified a stretch of 28 residues with the potential of forming an α-helical coiled-coil.
structure. The B30.2-like domain and coiled-coil region occur in some members of the protein family known as RBCC. Members of this family contain a tripartite motif of a RING finger, one or two B boxes, and a predicted coiled-coil domain (25, 26) (Fig. 4). Because we had not unequivocally identified the start codon, it was possible that the cloned GNIPt-h was not full length and that it lacked NH₂-terminal sequence corresponding to the RING finger and B box domains. This hypothesis was initially supported by the human EST data base in GenBank where we found several EST clones (GenBank accession numbers AI 139356, AI 290319, and AI 347404) containing sequences identical to 105 bp in the 5′-terminal region of the GNIPt-h cDNA, allowing us to extend the 5′ region of GNIPt-h. However, the extended region did not contain an in-frame ATG. From extensive 5′-RACE analyses, described in detail under “Experimental Procedures,” we were able to establish the existence of multiple GNIP transcripts that correspond to at least three different isoforms, which we designate GNIP1, GNIP2, and GNIP3.

The cDNA for GNIP1, the longest isoform, encodes a 511-amino acid protein with a predicted molecular mass of 60,630 Da that contains, according to SMART analysis, an NH₂-terminal RING finger domain followed by a B box domain in addition to the coiled-coil region and B30.2-like domain of the initial GNIPt-h clone (Fig. 4). GNIP2 is a truncated version of GNIP1, which lacks the NH₂-terminal RING finger and B box domains. GNIP3 would have a unique NH₂-terminal sequence, not present in GNIP1, and the COOH terminus that is identical in all three isoforms (Fig. 4). Note that we are predicting the GNIP3 transcript based only on amplification of the unique 5′ end and not the entire sequence (see “Experimental Procedures”). Also, we are assuming that the first ATG is the initiating codon, rather then one of two downstream ATGs. If the third ATG were the initiating codon, the product of translation would be the same as GNIP2. Searching GenBank with the GNIP1 protein sequence as query, we retrieved the sequence of the tripartite motif protein TRIM7 (GenBank accession number AF220032). The NH₂-terminal 206 residues of TRIM7 are identical to the NH₂ terminus of GNIP1 and contain RING finger and B box domains (Fig. 4). The COOH terminus of TRIM7 is short and contains 15 residues unique to TRIM7.

Mouse Homologues of GNIP—From the mouse EST data bank, we identified three homologous mouse clones. Two of these EST clones were obtained from ATCC (GenBank accession numbers AA517788 and AW012184) and sequenced. We found several sequencing errors in the published sequence of clone AA517788 and resubmitted the corrected 1,454-bp sequence (GenBank accession number AF396656). This sequence contains an ORF encoding a polypeptide of 303 residues, corresponding to GNIP2. The initiating ATG codon for the ORF is preceded by an in-frame stop codon suggesting that it corresponds to a full-length protein. The mouse GNIP2 is 93% identical to its human counterpart. The sequence of the second EST clone, AW012184, is identical to AF396656 only in the 3′ part that encodes GNIP2. The 5′ end of this clone is different and does not contain an in-frame terminating codon, suggesting that a larger isoform of mouse GNIP might exist.

Human GNIP Gene—We identified a clone of 183 kb in the unfinished high throughput genomic sequences that contained matches to GNIP. The clone (GenBank accession number AC008620) is derived from chromosome 5. Later, we found the GNIP gene in contig NT 006854.3, a working draft sequence segment of human chromosome 5. Exon-intron boundaries were established by comparison of the genomic sequences with GNIP and TRIM7 cDNA sequences. All the exon-intron boundaries conformed to the expected GT and AG sequences (Table 1). The gene for GNIP contains eight exons and seven introns (Fig. 5). The transcripts that we have identified can be explained by alternative splicing, by skipping exons, or by using alternate splice acceptor sites. Thus, splicing can generate smaller versions of exons 2, 3, and 4 (designated 2a, 3a, 3b, and 4a in Fig. 5). The cDNA for GNIP1 utilizes all exons, except exon 2, and exons 3 and 4 in this cDNA are represented by the exons 3a and 4a. We identified three different GNIP2 cDNAs that are distinguished by their 5′-UTR. These variants all contain exons 4a to 8 but have variant-specific regions from exons 2 and 3 (Fig. 5). GNIP3 cDNA is composed of five exons, from 4 to 8. The shortest isoform TRIM7 is derived from exons 2 and 3. The size of the exons varies from 23 bp (exon 5) to more than 1000 bp (exons 4 and 8). The first exon encompasses the 5′-UTR for GNIP1 and TRIM7 and the region encoding the RING finger and B box. The second exon encodes the 5′-UTR for GNIP2. Exon 3a is part of the 5′-UTR for GNIP2 but encodes part of the coiled-coil region in GNIP1. Exon 3b provides the 5′-UTR for GNIP2a. The entire exon 3 is present only in the cDNA for TRIM7 and encodes the coiled-coil region and the 3′-UTR. The larger version of exon 4 was found in the cDNA for GNIP3. This exon encodes 5′-UTR and part of coiled-coil motif. Exon 4a contains the initiating ATG codon for GNIP2 and encodes part of coiled-coil region in both GNIP1 and
GNIP2. The last exon, exon 8, encodes the B30.2-like domain and the 3'UTR for all isoforms of GNIP. Interestingly, another sequence, which is almost identical to the fragment of cDNA for GNIP1 (1–1516 bp, more than 99% identity), was reported to derive from chromosome 13 (contig NT009891.7). It is not yet clear if this represents a second GNIP gene or an annotation problem.

GNIP1 and GNIP2 Interact with Glycogenin—To confirm that the different isoforms of GNIP interact with glycogenin, we fused the cDNAs for GNIP1 and GNIP2 with the GAL4-AD in the pGADT7 vector and transformed yeast cells expressing GAL4-BD-glycogenin fusion protein. Both GNIP1 and GNIP2 were positive for interaction with glycogenin based on their ability to grow in the absence of adenine and histidine in the PJ69-4A yeast strain (data not shown). With GAL4-BD-glycogenin, GAL4-AD-GNIP1 and GAL4-AD-GNIP2 showed a 20- and 30-fold induction of β-galactosidase, respectively (data not shown). These results demonstrate that both isoforms interact with glycogenin.

### Table I

| Nucleotide sequence of intron-exon boundaries |
|-----------------------------------------------|
| bp    | bp    | bp    | bp    |
| Acceptor | Exon | Donor | Intron |
|--------|------|-------|--------|
| 1      | (>705) | . .caagGtacga...1 (<247) |
| ?      | 2 (>596) | . .ctcaagGtacga...2 (104) |
| ?      | 2a (>317) | . .cagagGttgctg...3 (>524) |
| ?      | 3 (>318) | . .tcagAgaaata...3 (>524) |
| ?      | Same 3a (96) | . .tcagAgaaata...3 (>524) |
| ?      | Same 3b (164) | . .tcagAgaaata...3 (>524) |
| 4 (>1050) | . .tcagAgaaata...3 (>524) |

*The sequence surrounding the intron-exon boundaries is shown with the acceptor and donor dinucleotide sequences shown by capital letters. The RING finger, B box, and B30.2 domains are underlined with a solid line. The potential coiled-coil domain is underlined with a dotted line. The TGA stop codon (*) is also indicated.
of GNIP specifically interact with glycogenin in yeast. Glyco-
genin and c-Myc-GNIP2 could be co-immunoprecipitated from
COS cells expressing both proteins (data not shown). We were
unable to demonstrate co-immunoprecipitation of c-Myc-
GNIP1 and glycogenin due to association of c-Myc-GNIP1 ex-
clusively with the pellet fraction from COS cells.

Expression of the GNIP Gene—To examine the tissue distri-
bution of GNIP expression, we probed a human multiple tissue
Northern blot (CLONTECH) using the entire cDNA fragment
from the two-hybrid clone. This probe detected major bands of
~3.4 kb in skeletal muscle and of 1.3 kb in placenta (Fig. 6).
The 3.4-kb band of significantly lower intensity was detected in
heart, brain, and pancreas. Additionally skeletal muscle con-
tained species of 1.4 and 5.8 kb. Heart and brain contained
species of 1.7 and 5.8 kb. The multiplicity of mRNA species is
consistent with the multiplicity of transcripts indicated by the
cDNA cloning.

GNIP2 Activates Glycogenin Self-glucosylation—In order to
examine the possible biological relevance of the interaction
between GNIP and glycogenin, we analyzed the effect of GNIP
on the self-glucosylation reaction catalyzed by glycogenin.
GNIP2 and glycogenin were produced as NH2-terminally His6-
tagged proteins expressed in E. coli. Expression of GNIP1
resulted in the accumulation of insoluble protein in bacteria,
and no soluble protein was obtained. Incubation of glycogenin
with UDP-[U-14C]glucose followed by SDS-PAGE and fluorog-
raphy revealed a single labeled polypeptide with ~42 kDa,
corresponding to His6-glycogenin (Fig. 7 ). In the presence of
GNIP2, incorporation of [14C]glucose into glycogenin was
markedly increased indicating that GNIP2 activates the reac-
tion. No labeling was associated with other proteins suggesting
that His6-GNIP2 is not an acceptor of [14C]glucose. Maximal
activation occurred at equal concentrations of both proteins,
suggesting a likely stoichiometry for the binding of glycogenin
to GNIP2 of 1:1. Glycogenin also transfers glucose from UDP-
glucose to low molecular weight acceptors, like n-dodecyl β-o-
maltoside. However, GNIP2 had no effect on glycogenin-medi-
ated incorporation of glucose into n-dodecyl β-o-maltoside
suggesting that the effect of GNIP2 was restricted to the self-
glucosylation reaction (data not shown). A more detailed char-
acterization of glycogenin activation by GNIP2 revealed that
the GNIP2 caused an almost 4-fold increase in Vmax with little
or no change in Km for UDP-glucose (Fig. 8).

DISCUSSION

We have identified a novel family of proteins, GNIPs, that
interact with glycogenin, a critical component of the glycogen
biosynthetic pathway. The search for interacting proteins also
selected glycogen synthase and glycogenin, whose ability to
bind glycogenin was already known. GNIP1 belongs to the
RBCC subgroup of RING finger proteins, which contain a sec-
ond zinc finger known as the B box, followed by a coiled-coil
domain (26, 27). COOH-terminal to the RBCC motif, GNIP1
contains a domain similar to the B30.2-like domain, which was
initially identified as a product of a coding sequence in the
chromosomal region containing the human major histocompat-
ability complex class I (21). Thus, GNIP1 is a member of the
more specific RBCC-B30.2 protein family (28). Proteins from
this family are involved in various processes including cell
growth and differentiation. This category includes the follow-
ing: the RET finger protein, RFP, which becomes oncogenic
when it recombines with the tyrosine kinase domain of the RET
protooncogene (29); putative transcription factors, the estro-
gen-responsive finger protein (30), EFP, and *Xenopus* nuclear factor 7 (31), Xnf7; the transcriptional regulator Staf-50 (32); the acid finger protein (33), AFP; the 52-kDa Sjorgen’s syndrome nuclear antigen A (34), SSA/Ro; and the RING finger B30 protein (35), RFB30, a protein containing the original B30.2 domain. Recently, several other members of the family have been identified, including testis RING finger protein (36), TERF; interferon-responsive RING finger protein 21 (37), RNF21; proteins associated with enterocyte differentiation (38), enterophilins. Several proteins with the B30.2-like domain are associated with pathological conditions. Mutations in the B30.2 domain of pyrin/marenostrin are thought to cause the autosomal recessive disease, familial Mediterranean fever (39, 40). Partial loss of the B30.2 domain in the midline 1 (MID1) protein caused by mutations is responsible for the Opitz G/BBB syndrome, characterized by developmental midline defects (41). Multiple alignment of protein sequences (using the ClustalW algorithm) indicated considerable homology of GNIP1 with the RBCC-B30.2 proteins, i.e. 36% identity with RFP, 35% identity with testis-abundant finger protein/Ring finger protein 23 (42), 32% identity with TERF, 32% identity with SSA/Ro, and 30% identity with Xnf7.

In attempts to amplify the 5’ end of GNIP cDNA obtained in two-hybrid screen, we found five distinct extensions. One would predict the cDNA for largest isoform of GNIP, GNIP1. Three distinct cDNA extensions predict three different 5’-non-coding regions for another protein isoform of GNIP, GNIP2 (Fig. 5). GNIP2 represents the COOH-terminal region of GNIP1, lacking the N-terminal RING finger and the B box domains (Fig. 4). The fifth cDNA extension generates the third isoform of GNIP, GNIP3. This isoform would contain a unique 26-residue sequence attached to the NH2 terminus of GNIP2. By searching GenBank™ with the GNIP1 protein sequence, we identified a fourth isoform of GNIP, TRIM7, which is identical in sequence to the NH2-terminal 206-residues of GNIP1 and contains a unique COOH-terminal tail of 15 residues. All variants are derived from one region of human chromosome 5, corresponding to the *GNIP* gene. Characterization of the human *GNIP* gene demonstrated a complex gene structure, with multiple differentially spliced transcripts. Some of the variation results from splicing occurring inside certain exons to produce smaller sub-exons such as 2a, 3a, 3b, and 4a (Fig. 5). The significance of this observation is not clear and could represent the existence of allelic variants.

The existence of multiple products derived from the *GNIP* gene is confirmed by other experiments. Northern blot analysis demonstrated transcripts of different sizes in skeletal muscle and one distinct species in placenta (Fig. 6). Significantly lower levels of GNIP expression were found in heart, brain, and pancreas. Consistent with our data, other studies demonstrated that one of the products of the *GNIP* gene, TRIM7, is selectively expressed in skeletal muscle in adult mice (43). However, TRIM7 is ubiquitously distributed in embryonic mouse tissues. Independent evidence for expression of GNIP2 in skeletal muscle was obtained from sequencing of a mouse EST clone (GenBank™ accession number AA517788) from the Barstead myotubes library. This clone contains the entire coding sequence for GNIP2 flanked by 5’-UTR and 3’-UTR, the latter including the poly(A) tail. Another EST clone (Gen-

**Fig. 7.** Effect of GNIP2 on self-gluco-sylating activity of glycogenin. Purified glycogenin (7 pmol) was incubated with UDP-[14C]glucose and the indicated amounts of recombinant GNIP2 at 30 °C for 20 min as described under “Experimental Procedures.” The reactions were terminated by adding SDS-loading buffer, and proteins were resolved by SDS-PAGE and autoradiograms prepared. The relative amounts of [14C]glucose incorporated into glycogenin were determined by optical density scanning of autoradiograms. The results are the means ± S.E. from four experiments. The inset shows the autoradiogram from one of these experiments.

**Fig. 8.** Effect of GNIP on UDP-glucose kinetics. Glycogenin (7 pmol) was incubated in the presence (open circles) or absence (closed circles) of GNIP2 (8 pmol) and five concentrations of UDP-[14C]glucose in the range of 1–20 μM. After incubation at 30 °C for 5 min the aliquots were spotted onto P81 chromatography paper for quantitation by scintillation counting.

**GNIP2, pmol**

| GNIP2, pmol | Glycogenin activity (% of control) |
|------------|-----------------------------------|
| 0          | 100                              |
| 0.34       | 80                               |
| 2.1        | 60                               |
| 8.2        | 40                               |

**GNIP2, pmol**

| [14C-UDPG] μM | Glucose Incorporation (μmol/mg/min) |
|--------------|-------------------------------------|
| 0            | 0                                   |
| 5            | 1                                   |
| 10           | 2.5                                 |
| 15           | 3.5                                 |
| 20           | 4.0                                 |

**FIG. 7.** Effect of GNIP2 on self-gluco-sylating activity of glycogenin. Purified glycogenin (7 pmol) was incubated with UDP-[14C]glucose and the indicated amounts of recombinant GNIP2 at 30 °C for 20 min as described under “Experimental Procedures.” The reactions were terminated by adding SDS-loading buffer, and proteins were resolved by SDS-PAGE and autoradiograms prepared. The relative amounts of [14C]glucose incorporated into glycogenin were determined by optical density scanning of autoradiograms. The results are the means ± S.E. from four experiments. The inset shows the autoradiogram from one of these experiments.

**FIG. 8.** Effect of GNIP on UDP-glucose kinetics. Glycogenin (7 pmol) was incubated in the presence (open circles) or absence (closed circles) of GNIP2 (8 pmol) and five concentrations of UDP-[14C]glucose in the range of 1–20 μM. After incubation at 30 °C for 5 min the aliquots were spotted onto P81 chromatography paper for quantitation by scintillation counting.
Bank™ accession number AW012184) from mouse kidney might represent a partial sequence of the GNIP1 isoform. Interestingly, both full-length and truncated versions were found for other members of the RBCC-B30.2 protein family (37, 43). For example, the gene for RNF21 generates at least three isoforms, due to alternative splicing (37). Expression of one specific form in HeLa cells was dramatically up-regulated by interferon. It is possible that expression of individual forms of GNIP in skeletal muscle is selectively regulated by different interferon. In-
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