SKP1 is involved in the ubiquitination of certain cell cycle and nutritional regulatory proteins for rapid turnover. SKP1 from *Dictyostelium* has been known to be modified by an oligosaccharide containing Fuc and Gal, which is unusual for a cytoplasmic or nuclear protein. To establish how it is glycosylated, SKP1 labeled with [3H]Fuc was purified to homogeneity and digested with endo-Lys-C. A single radioactive peptide was found after two-dimensional high performance liquid chromatography. Analysis in a quadrupole time-of-flight mass spectrometer revealed a predominant ion with a novel mass. Tandem mass spectrometry analysis yielded a set of daughter ions which identified the peptide and showed that it was modified at Pro-143. A second series of daughter ions showed that Pro-143 was hydroxylated and derivatized with a potentially linear pentasaccharide attached to hydroxylated Pro-143. The attachment site was confirmed by Edman degradation.

Gas chromatography-mass spectrometry analysis of trimethylsilyl-derivatives of overexpressed SKP1 after methanolysis showed the HexNAc to be GlcNAc. Exoglycosidase digestions of the glycopeptide from normal SKP1 and from a fucosylation mutant, followed by matrix-assisted laser desorption time-of-flight mass spectrometry analysis, showed that the sugar chain consisted of 6-O-(2→1)-L-Fucα1→3-2-D-Galβ1→3GlcNAc. Matrix-assisted laser-desorption time-of-flight mass spectrometry analysis of all SKP1 peptides resolved by reversed phase-high performance liquid chromatography showed that SKP1 was only partially hydroxylated at Pro-143 and that all hydroxylated SKP1 was completely glycosylated. Thus SKP1 is variably modified by an unusual linear pentasaccharide, suggesting the localization of a novel glycosylation pathway in the cytoplasm.

SKP1 is found in a multiprotein complex with cullin (a cdc53 homologue) and an F-box-containing protein to form the SCF complex, named as an acronym of the participating proteins. When this complex contains an E2 enzyme, it is responsible for ubiquitinating various target proteins, depending on the identity of the F-box protein. Targets for subsequent degradation identified in *Saccharomyces cerevisiae* include cell cycle proteins such as the S-phase kinase inhibitor SIC1 and G3 cyclins, and proteins specific to the nutrition of the cell (1–4). The SCF complex has also been implicated in phosphorylation of kinetochores (5), and another distantly related complex affects mRNA metabolism (6). An SCF complex with Cyclin A and Cdk2 has been detected in mammalian cells, and its abundance appears increased in transformed cells (7, 8). SKP1 itself is abundantly and dynamically expressed in the mouse embryo (9) and central nervous system including postmitotic neurons (10) and at very high concentrations in the inner ear organ of Corti (11, 12), where it comprises up to 5% of total protein in the cytoplasm. The expression of several SKP1 genes in plants appears to be governed by morphogenetic boundaries (13). Thus SKP1 is expressed ubiquitously in eukaryotes, and its role may be to facilitate the selection of other proteins for specific posttranslational modification.

Binding of SKP1 to different proteins may be regulated structurally because it is encoded by multiple genes in multicellular organisms (1, 11, 14–16). SKP1 structure is also altered by glycosylation in *Dictyostelium discoideum* (17, 18), which may regulate its activity. Because complex glycosylation of cytoplasmic/nuclear proteins is unusual, we embarked on a study to establish the structure of the carbohydrate modification as a first step in investigating its function.

To determine the structure and site of attachment of the previously described SKP1 fuco-oligosaccharide(s) (17, 18), mass spectrometric approaches were employed. Key to the success of the this methodology was the newly developed Q-TOF MS2 (19, 20), which permitted MS-MS studies to be performed on the picomole quantities of material available from native sources. This recently designed instrument comprises a quadrupole with collision cell followed by an orthogonal acceleration TOF analyzer that confers a high degree of sensitivity and accuracy to the mass measurements. The ability of this instrument to sequence both the peptide and oligosaccharide chains of an SKP1 fucoglycopeptide suggested that it consists of a linear pentasaccharide attached to hydroxylated Pro-143. The sugar sequence was established from exoglycosidase studies using MALDI-TOF MS and sugar analyses on genetically overexpressed material. The protein attachment site was then confirmed by Edman degradation.

These results reinforce the model that complex O-linked glycosylation occurs in the eukaryotic cytoplasmic compartment. Whereas there has been much circumstantial evidence to

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support this model (21), it has remained controversial (22). In contrast, simple glycosylation, in the form of GlcNac O-linked to residues of Ser or Thr, is well established in this compartment (23). The sugar structures that have been described to date on cytoplasmic/nuclear proteins are generally distinctive from those produced in the secretory pathway, suggesting that there may be fundamental differences in the biogenesis and function of these modifications.

**Experimental Procedures**

**Construction of Strain HW120**—A full-length cDNA from the *fpa1* gene with a decapeptide encoding the human c-myc epitope (24) inserted between codons 161 and 162 near the C terminus was prepared by PCR and cloned into pT7Blue (Novagen). The cDNA was subcloned into pVHEHΔATG (25) under the control of the inducible discoi din promoter and the actin-8 terminator. The plasmid was transformed into *D. discoideum* by a CaPO₄ precipitation method and selected in the presence of 15 μg/ml G418 (26). Strain HW120 was a clone that produced SKP1-c-myc at high levels after growth in 120 μg/ml G418, based on Western blot analysis with monoclonal antibody 9E10 against the c-myc epitope. SKP1-c-myc contained two missense mutations, T194C and A305G resulting in the amino acid substitutions I34T and D71G, introduced by the PCR reaction and confirmed by MS analysis of its peptides (data not shown).

**Purification of SKP1**—-*D. discoideum* strains Ax3, HL250, or HW120 were grown to stationary phase in HL-5 medium (17). Cells were filtered-lysed, and an S100 fraction was prepared by ultracentrifugation (17). SKP1 from Ax3 and HL250 were fractionated into pools I and II by DEAE-anion exchange chromatography (14); SKP1-c-myc from strain HW120 eluted after pool-II. Each pool was subsequently purified by phenyl-Sepharose and monoclonal antibody 3F9 affinity chromatography (14). For metabolic labeling, a 200-ml culture of Ax3 cells was grown for three generations in 0.05 mM[^3]H[Fuc] in HL-5. SKP1 was isolated in the same manner except the phenyl-Sepharose step was omitted, and purified radiolabeled SKP1 was pooled with unlabeled SKP1. In some cases, SKP1 was reduced and carboxamidomethylated (14) and further purified on a C₈/C₁₈ (PC 3.2/3) RP-HPLC column on an Amersham Pharmacia Biotech SmartSystem HPLC (14). The peak that contained >80% of total SKP1 was examined further. Recombinant SKP1 (fpa1) containing an N-terminal oligo-His tag was isolated from *Escherichia coli* as described (27) and further purified on an RP column.

**Monosaccharide Composition Analysis**—2 nmol of mannitol was added as an internal standard to 1.5 nmol of SKP1-c-myc. The sample was subjected to methanolysis, re-N-acetylation, and TMS-derivitization as described (28) and analyzed in splitless mode on a 0.32 mm x 30 m SPB-1 column (Supelco) on a Shimadzu QP-5000 GC/MS workstation. Peaks were analyzed with selected ion monitoring at m/z 204 for the neutral sugars and m/z 173 for the N-acetylated amino sugars.

**Exoglycosidase Digestion of SKP1 Glycopeptides**—Carboxamidomethylated SKP1 was digested with endo-Lys-C from *Achromobacter lyticus* (Wako Chemicals, Richmond, VA) in the presence of 2 m urea in 0.2 m Tris-HCl, pH 7.4, using an enzyme:substrate ratio of 1:200 (mol/mol), at 30 °C for 18 h. Peptides from metabolically labeled SKP1 were fractionated on a Superdex Peptide HR10/30 column (Amersham Pharmacia Biotech) in 6 m urea in 50 mM NaCl, 50 mM Tris-HCl, pH 7.2, on an LKB GTH PFC system at 0.5 ml/min. Radioactive fractions were applied to a 4.6 x 150 mm, 3.5-μm particle C₁₈ column (Zorbax) and eluted with a gradient of 5% (v/v) MeCN in 0.1% (v/v) trifluoroacetic acid to 40% MeCN in 0.085% trifluoroacetic acid at 1 ml/min. Peptides (20 pmol) were subjected to Edman degradation on an ABI model 494 Procise sequencer. Nonradiolabeled peptides were fractionated directly on the C₁₈ or a C₇/C₁₈ (PC 2.1/10; Amersham Pharmacia Biotech) RP-column.

**MALDI-TOF MS**—Samples were mixed with an equal volume of saturated a-cyan-o-4-hydroxycinnamic acid (Alrich) in 70% MeCN. 1 μl (2-3 pmol) was deposited on a sample plate and air dried. Spectra were collected on a PerSeptive Biosystem Voyager RP MALDI-TOF MS operated in the positive ion, linear mode.

**Q-TOF Mass Spectrometry**—Samples from RP fractions were introduced directly into the Q-TOF MS (Micromass, UK) via a nanospray device (19, 20). Primary and secondary ion spectra were collected in the positive ion mode.

**Exoglycosidase Digestion of SKP1 Glycopeptides**—15 pmol of glycopeptide from RP fractions were partially dried by vacuum centrifugation, diluted to a final volume of 2.5 μl with the glycosidase preparation, incubated for 18 h at 37 °C, and processed for MALDI-TOF MS. Nonsusceptible substrates yielded no new ions after digestion. Green coffee bean α-galactosidase (Gala1→4>2,3) from Boehringer Mannheim was further purified as described (29) and used at 10 milliunits/μl in 50 mM ammonium phosphate, pH 6.0. Recombinant α-galactosidase (Gala1→3) from Glyko (Novato, CA) was used at 10 microunits/μl in 20 mM ammonium phosphate, pH 6.0. *Xanthomomas manihotis* α-galactosidase (Gala1→4) from New England Biolabs was used at 10 microunits/μl in 20 mM ammonium phosphate, pH 6.0. Sweet almond β-glucosidase (Glu/Gal/Fucβ→) from Boehringer Mannheim was used at 10 milliunits/μl in 20 mM ammonium phosphate, pH 5.0. Bovine kidney α-fucosidase (Fucα1→2/3/4) from Boehringer Mannheim was used at 2.5 milliunits/μl in 20 mM ammonium phosphate, pH 5.0. X. manihotis α-fucosidase (Fucα1→2) from New England Biolabs was used at 100 milliunits/μl together with purified green coffee bean α-galactosidase in 50 mM ammonium phosphate, pH 6.0. Bovine kidney β-galactosidase (Galβ1→3/4) from Oxford GlycoSciences (Abingdon, UK) was used at 4 milliunits/μl in 25 mM ammonium acetate, pH 4.0. Recombinant β-galactosidase (Galβ1→3/4) from Glyko was used at 4.8 milliunits/μl in 20 mM ammonium phosphate, pH 5.0. *X. manihotis*
β-galactosidase (Galβ1–3) from New England Biolabs was used at 4.8 milliunits/μl in 20 mM ammonium phosphate, pH 5.0. Jack bean β-HexNAcase (GlcNAc/GalNAcβ3) from V-Labs was used at 6.5 milliunits/μl together with bovine kidney β-galactosidase in 20 mM ammonium phosphate, pH 5.0.

Mild Acid Hydrolysis of the Fucoglycopeptide—The peptide fraction was partially dried in a vacuum centrifuge, reconstituted in 10 μl of 0.05 M trifluoroacetic acid, and incubated at 95 °C for up to 6 h.

RESULTS

Isolation of the SKP1 Fucoglycopeptide—To investigate the glycosylation of SKP1, normal cells (strain Ax3) were metabolically labeled with [3H]Fuc, and SKP1 was purified to homogeneity. Reduced and alkylated SKP1 was digested with endo-Lys-C and fractionated on a Superdex peptide gel filtration column (Fig. 1A). Radioactivity eluted as a single peak. When fraction 23 was separated on a C8 RP-HPLC column, radioactivity again eluted in a single peak, which was centered at fraction 35, did not absorb at 280 nm, and contained 30% of the original radioactivity (Fig. 1B). These results suggested that SKP1 contained only a single fucoglycopeptide.

Q-TOF MS Analysis—A parallel experiment using nonradioactive Fuc yielded material for MS analysis. The HPLC fraction (number 35) containing the putative fucoglycopeptide was subjected to tandem mass spectrometry on a Q-TOF mass spectrometer. Analysis of a few picomoles in the MS-only mode gave a major [M+3H]3+ signal at m/z 829.42 (Fig. 2, including inset). Note the resolved natural abundance 13C isotopes at m/z 829.70 and 830.03 showing that this signal is triply charged. It therefore derives from a molecule of mass 2485 Da. A series of doubly charged ions was also apparent in the spectrum, separated by sugar mass differences (m/z 1244, 1163, 1081, 1008, and 927).

The ion at m/z 1244 is the [M+2H]2+ ion corresponding to the [M+3H]3+ at m/z 829.42, and the mass differences from this correspond to intervals of Hex, Hex, Fuc, and Hex, respectively. MS/MS of the m/z 829 [M+3H]3+ ion using argon gas and 50 eV collision energy produced the spectrum shown in Fig. 3. Interestingly, the spectrum shows the formation (from m/z 829) of a number of doubly charged ions at m/z 826, 927, 1009, 1081 and 1163 separated by sugar mass differences of HexNAc, Hex, Fuc, and Hex, respectively, suggesting a stepwise stripping of these sugars from a linear oligosaccharide attached to the glycopeptide. These data confirm that, in the MS spectrum shown in Fig. 2, the labeled doubly charged ions were formed in that case by cone-voltage-induced fragmentation from a true quasimolecular ion at m/z 829. The doubly charged ion at m/z 826 (Fig. 3) showed no evidence of further sugar loss and is interpreted as the remaining peptide backbone. This is confirmed by the presence of a major singly charged quasimolecular ion signal at m/z 1651 [M+H]+ for the peptide. A search for this mass in the gene-derived sequence finds nothing, but the peptide identity can be determined from the presence of N- and C-terminal sequence ions (30) (b and y0, respectively) in the low mass range of the spectrum (Figs. 3 and 4A) to give the sequence . . . EEQL/IRK. Note the relatively high intensity of the m/z 544 isotope peak at m/z 545 (Fig. 4A) showing that the Q residue is partially deamidated. This is observed in all ions containing that residue and also accounts for the high intensity of the 1651.79 satellite to the quasimolecular ion signal in Fig. 3. The
partial sequence determined from the C-terminal \((y^0)\) ions is further complemented by N-terminal \((b)\) ions at \(m/z\) 230, 377, and 478 corresponding to a \(230\)-F-T sequence. The sequence ion data thus lock the peptide portion of the molecule onto residues 139–151 in the sequence \((NDFTPEEEEQIRK)\) except that the mass found in the Q-TOF MS/MS collision spectrum is 16 Da higher than that predicted for the peptide \((1635 [M+H]^+)\). This is interpreted as a posttranslational hydroxylation of one of the amino acids present, and this could not be at the C-terminal Lys residue, already assigned in the fragmentation data.

Screening the higher mass fragments in the spectrum allows a clear assignment of where the 16-Da increment is attached via the signals at \(m/z\) 1060 and 1173 in Fig. 4B. These show that the Pro C-terminal sequence ion, \(y^9\), is 16 Da higher than the theoretical value for the sequence \((1057 Da)\), showing that it is a HyPro in this position. The signals at \(m/z\) 1274 and 1421 complete a series showing -F-T-P(OH)- in the sequence.

Importantly, Fig. 4B also allows assignment of the attachment site of the carbohydrate to the peptide. The signal at \(m/z\) 1376 is a HexNAc residue away from the \(m/z\) 1173 ion, showing that the carbohydrate is attached via a HexNAc residue to the HyPro and not, as may have been expected, to the Thr residue. A further signal at \(m/z\) 1477 extends this ion series to the \(y^{10}\) sequence ion \(TP(OH)\)EEEEEQIRK substituted with HexNAc on the HyPro. The signal at \(m/z\) 1538 corresponds to the \(y^9\) ion with a Hex-HexNAc substituent, and the sugar sequence data assigned from the doubly charged ions at \(m/z\) 826, 927, 1008, 1081, 1163, and 1244 (Figs. 2 and 3) now overlaps the singly charged MS/MS glycopeptide data giving confirmation of the sequence. MS/MS data on several of the cone-voltage-induced doubly charged glycopeptide fragment ions also corroborated the above interpretation (data not shown).

A mild trifluoroacetic acid hydrolysis experiment on a remaining small quantity of sample showed the disappearance of the \(m/z\) 829 triply charged signal together with the other doubly charged ions, excepting \(m/z\) 1008 and a weak triply charged signal at \(m/z\) 672, suggesting, as do the MS/MS data, that the saccharide is a linear molecule with Fuc mid-chain.

Taking all the MS and MS-MS data into consideration the structure of the fucoglycopeptide was assigned as \(NDFT(\text{Hex}^3\text{Hex}^3\text{Fuc}^3\text{Hex}^3\text{HexNAc})\) hydroxyPEEEEQIRK. Subsequent Edman degradation sequencing on a 20-pmol sample confirmed the entire sequence of 13 residues, except for a blank at cycle 5, expected to be Pro.

**MALDI-TOF MS Analysis**—MALDI-TOF MS of the fucoglycopeptide provided corroborative evidence for the carbohydrate sequence predicted from the Q-TOF experiments. The MALDI spectrum contained a major \(M+H^+\) signal at \(m/z\) 2487 (Fig. 5A) which was equivalent to the triply charged ion at \(m/z\) 829.70 in the Q-TOF spectrum. In addition, a series of low abundance ions were observed at \(m/z\) 2326, 2164, 2017, 1855, and 1652 consistent with sequential loss of Hex, Hex, Fuc, Hex, and HexNAc, respectively. The low abundance ions appeared to be fragmentation products of the \(m/z\) 2487 ion resulting from similar frequency of scission of each of the glycosidic linkages \((31)\) rather than incompletely glycosylated species, because less glycosylated peptides eluted later from the RP column (see below). The \(m/z\) 2487 ion was not seen in a similar analysis of RP-fractionated peptides (see below) of recombinant SKP1 isolated from *E. coli* (data not shown), showing that it was specific to the protein expressed in *Dictyostelium*. 
Sugar Composition Analysis—To determine its monosaccharide composition, SKP1 was isolated from the S100 fraction of a D. discoideum strain (HW120) genetically modified to over-express, under the control of the inducible discoidin promoter, a form of the protein with a c-myc-epitope tag near its C terminus. The sugars of purified SKP1-c-myc were examined by GC/MS after methanalysis, re-N-acetylation, and formation of TMS derivatives. To achieve the sensitivity required to analyze 1.5 nmol of SKP1-c-myc, splitless injection and selected ion monitoring were employed. Fuc (0.33 nmol), Gal (0.70 nmol), and GlcNAc (0.32 nmol) were the three most abundant sugars detected, and their low levels indicated that overexpressed SKP1 was under-glycosylated. No GalNAc was detected. Fuc, Gal, and Xyl were previously detected after 2 m trifluoroacetic acid hydrolysis (18), but GlcNAc was not. The stronger acid conditions of methanalysis were probably required to liberate GlcNAc from its linkage with HyPro, and Xyl is not found in the fucoglycopeptide according to the MS results. Thus the GC/MS data showed that the deoxyHex residue identified by MS was l-Fuc, as suggested by metabolic labeling, and showed that all three Hex residues were Gal and the HexNAc was GlcNAc.

Exoglycosidase Digestions—To assign linkages, the fucoglycopeptide was treated with exoglycosidases and analyzed by MALDI-TOF MS. The mass of the glycopeptide was reduced by 2 m trifluoroacetic acid hydrolysis (18), but GlcNAc was not. The stronger acid conditions of methanalysis were probably required to liberate GlcNAc from its linkage with HyPro, and Xyl is not found in the fucoglycopeptide according to the MS results. Thus the GC/MS data showed that the deoxyHex residue identified by MS was l-Fuc, as suggested by metabolic labeling, and showed that all three Hex residues were Gal and the HexNAc was GlcNAc.

Taking all of the evidence together, the sugar sequence was assigned as p-Galp1→6-p-Galp1→κ-Fucp1→2-p-Galp1→3-p-GlcNAc→HyPro-143. Configurations and the assignment of Gal and Fuc as pyranose forms were based on the exoglycosidase specificities. This structure was derived from about 50 pmol of purified fucoglycopeptide. Methods which require substantially larger amounts of sugars will be necessary to determine the exact linkage of the second α-linked Gal residue and the configuration of the GlcNAc→HyPro linkage.

Glycosylation Heterogeneity—Detection of the unmodified peptide in the fucosylation mutant suggested that hydroxylation of Pro-143, the first step in the glycosylation pathway, is rate-limiting and possibly regulatory. Similarly, MALDI-TOF MS analysis of RP fractions from endo-Lys-C released peptides from pool-I of SKP1 from normal cells yielded ions corresponding only to the pentasaccharide—peptide (m/z 2487) and unmodified peptide139→152 (m/z 1637) (data not shown). It remains to be determined whether Pro-143 hydroxylation occurs on products of both SKP1 genes, as each are present in pools-I and -II, as shown previously by Edman degradation (14) and confirmed here by MS (data not shown). To characterize the glycosylation pathway further, SKP1-c-myc was expressed at an elevated level in strain HW120. Endo-Lys-C-generated peptides were fractionated by RP-HPLC and analyzed by MALDI-TOF MS, yielding abundant [M+H]^+ ions of 2487, 2327, 2165, and 1636 in successive fractions (data not shown). These corresponded to multiple glycoforms, including the full-length pentasaccharide, the pentasaccharide minus one or two of the outer α-linked Gal residues, the hydroxylated but unglycosylated peptide, and the unmodified peptide. These findings were consistent with the monosaccharide composition results shown above. 1) Fuc and GlcNAc were present at equal levels, as expected because no mono- and disaccharide intermediates were detected. 2) The Gal:Fuc ratio was 2:1, indicating that there is on average one α-linked Gal (the other being β-linked), which correlated with the detection of glycopeptides containing 0, 1, or 2 outer Gal residues. 3) Only 22% of the protein was glycosylated, which correlated with the high levels of unmodified and hydroxylated forms of peptide 139→152 detected. The results indicated that, secondary to Pro hydroxylation, attachment of the reducing terminal GlcNAc and the outer α-linked Gal residues were the most rate-limiting, suggesting that the enzymes that add these sugars may potentially regulate the structure of the Pro-143 glycan at other stages of the life cycle.

Fig. 4. Parts of the MS/MS spectrum shown in Fig. 3 expanded to show fragment ions more clearly. A, low mass region containing peptide-derived fragment ions. B, higher mass region containing peptide and glycopeptide fragment ions.
The mass spectrometric analyses suggested that the SKP1 glycan consists of a linear pentasaccharide attached to a HyPro at position 143. The attachment site was confirmed by Edman degradation. The exoglycosidase studies and sugar analyses confirmed the linear model and showed the sequence to be \( \text{d-Galp} \rightarrow 6 \rightarrow \text{d-Galp} \rightarrow 1 \rightarrow \text{L-Fuc} \rightarrow 1 \rightarrow \text{GlcNAc} \rightarrow \text{HyPro-143} \). Substantially greater amounts of the fucoglycopeptide will be necessary to determine the linkage position of the second \( \alpha \)-linked Gal residue, and the configuration of the GlcNAc \( \rightarrow \) HyPro linkage. The core trisaccharide, \( \text{Fuca} \rightarrow 2 \rightarrow \text{Gal} \beta 1 \rightarrow 3 \rightarrow \text{GlcNAc} \), is equivalent to blood group H (type 1) expressed by mammalian cells (32). Although internal Fuc linkages have been found in glycoproteins (33, 34), the outer Gal \( \rightarrow \) 6Gal\( \alpha 1 \rightarrow \) Fuc cap structure has not been previously described. However, this sugar chain is not immunogenic in mice (18), unlike other Dictyostelium sugar protein conjugates (35, 36), suggesting that a similar structure may be expressed in mammals. The linkage amino acid, hydroxyproline, is possibly 4-hydroxylated based on the specificity of a SKP1:UDP-GlcNAc GlcNAc-transferase activity that has been detected and partially purified. 2 4-hydroxylation is phylogenetically ubiquitous and 4-OH-Pro has been found to be derivatized with Ara or Gal in plants and algae (37, 38), but substitution by GlcNAc has not been described previously. The double-negative charge previously attributed to the sugar moiety after attempted \( \beta \) elimination (17) may have resulted from base-catalyzed scission of the adjacent E\( \rightarrow \)E peptide bond, as sugar \( \rightarrow \) HyPro linkages are known to be alkali-resistant (37, 38). GlcNAc was not previously detected in SKP1 (18), probably because methanalysis is more effective than 2 m trifluoroacetic acid in causing its release from HyPro.

The aforementioned structures homologous to the SKP1 oligosaccharide are synthesized in either the rER or the Golgi apparatus and then expressed on the cell surface or secreted. However, SKP1 is not secreted, but rather is located and functions in the cytoplasm and nucleus (1–13, 17, 18). This implies that known enzymes directing the synthesis of the homologous structures would not be accessible to SKP1, unless SKP1 transiently visits the lumen of the secretory pathway.

Current evidence suggests that SKP1 is partially modified in the cytoplasm by a novel biosynthetic pathway. The enzyme which adds Fuc is likely to be the previously characterized cytosolic fucosyltransferase (cFTase). The cFTase fucosylates SKP1 in vitro with a submicromolar \( K_m \) (27) and catalyzes formation of the same Fuc linkage on the same acceptor disaccharide (27, 39), as the present results show occur naturally in SKP1. The cFTase is likely to reside in the cytoplasmic compartment of the cell as it was purified from the cytosolic fraction, and its submicromolar \( K_m \) for GDP-\( \beta \)Fuc is more characteristic of cytoplasmic compared with Golgi glycosyltransferases (27). Recent studies on Pro hydroxylase and GlcNAcTase activities that modify overexpressed SKP1-\( \text{c-myc} \) and synthetic peptides suggest that they also reside in the cytoplasmic compartment. 3 These enzymes may constitute a hitherto unrecognized complex pathway of O-glycosylation localized in the cytoplasm, not the secretory pathway, that attaches sugars to SKP1 incrementally rather than en bloc. Although glycosylation heterogeneity was not observed in SKP1 from cells at the end of the growth phase, accumulation of incompletely glycosylated chains in the overexpression strain raises the possibility of the expression of glycoforms at Pro-143 at other stages of the life cycle.

Pro-143 is located within the highly conserved C-terminal region of SKP1. This implies that it is likely to be a novel glycosylation site, and a similar site in other proteins might be a target for future investigation.

**FIG. 5.** MALDI-TOF MS analysis of SKP1 glycopeptides. A–D, spectra of the fraction 35 fucoglycopeptide from Fig. 1, isolated from the normal strain Ax3. A, in addition to the primary ion, a series of low abundance fragment ions corresponding to selective glycosidic scissions of the glycan chain are noted. B, treatment of the fraction 35 fucoglycopeptide with \( X. \) manihotis \( \alpha 1 \rightarrow 3/6 \)-galactosidase. C, treatment of the fraction 35 fucoglycopeptide with green coffee bean \( \alpha \)-galactosidase. D, treatment of the fraction 35 fucoglycopeptide with green coffee bean \( \alpha \)-galactosidase and \( X. \) manihotis \( \beta 1 \rightarrow 3 \)-galactosidase. G, a later eluting RP-HPLC fraction containing the unmodified peptide. Structures suggested by the experimental result shown and other results are given in each panel in brackets.

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2 P. Teng-umnuay and C. M. West, unpublished data.
The structural evidence shown here adds SKP1 to the short list of examples that complex glycosylation does in fact occur on cytoplasmic and nuclear proteins, as has often been postulated based on indirect evidence (21, 22). It has been well documented in the past decade that many cytoplasmic/nuclear proteins are monoglycosylated by GlcNAc on Ser or Thr residues. However, the only generally accepted examples of oligoglycosylation are an incompletely defined, pan-eukaryotic Glc-1-PO$_4$ modification of phosphoglucomutase (40), also known as parasumin, and glycogenin, the primer for glycogen (41). The novel GlcNAc–HyPro linkage in SKP1 is distinct from the GlcNAc–Ser/Thr (23), Glc–Tyr (41), and possible Man–Ser/Thr (40) linkages found in the other cytoplasmic/nuclear proteins, and has not been detected on proteins modified in the secretory pathway. Though other proteins in the cytoplasmic fraction appear to be metabolically labeled by [3H]Fuc, SKP1 appears to be a major recipient of cFtase-mediated fucosylation both in vivo and in vitro (17, 18). Further studies are required to determine whether the other fucoproteins in this fraction reside in the cytoplasmic/nuclear compartment prior to cell lysis. The availability of the newly designed Q-TOF MS is expected to make it more practical to investigate the posttranslational modifications of other lower abundance intracellular proteins, which must be isolated from natural sources to analyze their posttranslational modifications.

Cytoplasmic glycosylation can have dramatic consequences, as highlighted by a Clostridial enzyme toxin that applies GlcNAc to a specific Thr residue of cytoplasmic rho and cde42 proteins resulting in major effects on the actin cytoskeleton (42). The function of the SKP1 pentasaccharide modification is not likely to involve competition with phosphorylation as proposed for the simple GlcNAc monosaccharide modification of many cytoplasmic/nuclear proteins (23). Instead, it may serve as a ligand for a cytoplasmic/nuclear carbohydrate-binding protein (43, 44) or as a steric shield. The observation that the pentasaccharide modification appears to be completed on all pool I and pool II SKP1 proteins whose Pro-143 residues are hydroxylated supports the model that it is a ligand for a receptor, which must be structurally rich and constant. SKP1 subpopulations differing with respect to the pentasaccharide–HyPro modification might vary in their ability to interact with specific F-box containing proteins, thereby potentially regulating ubiquitination or phosphorylation of selected target proteins in response to e.g. changes in the nutritional, differentiation, or cell cycle status of the cell (1–13). The new knowledge of glycan structure and attachment now renders the function of SKP1 glycosylation susceptible to genetic investigation.