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Analysis of Skp1 glycosylation and nuclear enrichment in *Dictyostelium*

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Skp1 is a subunit of SCF-E3 ubiquitin ligases and other protein complexes in the nucleus and cytoplasm of yeast and mammalian cells. In *Dictyostelium*, Skp1 is partially modified by an unusual pentasaccharide *O*-linked to hydroxyproline143. This modification was found to be susceptible to known prolyl hydroxylase inhibitors based on M_r-shift analysis using SDS–polyacrylamide gel electrophoresis/Western blotting. In addition, *Dictyostelium* Skp1 consists of 2 genetic isoforms, Skp1A and Skp1B, which differ by a single amino acid and appear to be expressed throughout the life cycle based on reverse-transcription polymerase chain reactions. The significance of these structural variations was examined by expressing myc-tagged Skp1s and mutants that lacked the glycosylation site. Gel-based M_r-shift studies showed that Skp1A and Skp1B are both nearly completely glycosylated during growth and early development, and mass spectrometry of glycopeptides showed that they were glycosylated similarly. Skp1 expressed later in prespore cells was not glycosylated, unlike bulk Skp1 persisting from earlier in development, but became glycosylated after return to growth medium. Skp1A and Skp1B were each concentrated in the nucleus and regions of the cytoplasm, based on immunofluorescence localization. However, when Skp1 glycosylation was blocked by mutation, prolyl hydroxylase inhibitors, or expression in prespore cells, nuclear concentration of Skp1 was not detected. Furthermore, nuclear concentration occurred in a mutant that attached only the core disaccharide to Skp1. Overall, there was no evidence for differential Skp1 isoform expression, glycosylation variants in the bulk Skp1 pool, or regulation of nuclear localization. However, these studies uncovered evidence that the glycosylation pathway is developmentally regulated and can function posttranslationally, and that core glycosylation is required for Skp1's nuclear concentration.

Key words: α,α′-dipyridyl/cytoplasmic glycosylation/ethyl 3,4-dihydroxybenzoate/hydroxyproline/prolyl hydroxylase/ubiquitin ligase

Introduction

Skp1 is a subunit of the SCF class of E3 ubiquitin ligases that is responsible for targeting proteins for ubiquitination and degradation (Williams *et al.*, 1999; Laney and Hochstrasser, 1999; Koepp *et al.*, 1999; Deshaies, 1999). Genetic and biochemical studies show that Skp1 directly contacts the F-box protein and cullin-1, which is in turn linked to an E2 enzyme. Different SCF complexes, which vary in their F-box proteins, are specific for the ubiquitination of individual cell cycle regulatory proteins, transcriptional factors, and other signaling proteins. F-box proteins and a cullin have recently been identified in *Dictyostelium* (Chung *et al.*, 1998; Ennis *et al.*, 2000; Nelson *et al.*, 2000). The precise function of Skp1 has yet to be elucidated.

Target substrates for SCF E3 ubiquitin ligases reside in both the nucleus and the cytoplasm. Proteasomes, which are the ultimate destination of proteins ubiquitinated by the SCF E3 complex, are also found in both the nucleus and cytoplasm (Schauer *et al.*, 1993). Skp1 is enriched in the nucleus of cultured mammalian cells based on immunolocalization studies (Freed *et al.*, 1999; Gstaiger *et al.*, 1999). Genetic and biochemical studies show Skp1 in yeast kinetochores (reviewed in Deshaies, 1999), where it apparently belongs to a protein complex distinct from the SCF complex. In the cytoplasm, Skp1 is concentrated in the mammalian centrosome (Freed *et al.*, 1999; Gstaiger *et al.*, 1999) and partially associated with the microsomal fraction in a salt-sensitive fashion in *Dictyostelium* (Kozarov *et al.*, 1995). A 2-hybrid screen has suggested an association with the cytoplasmic actin-binding protein coronin (Uetz *et al.*, 2000). The mechanism by which Skp1 is distributed between the nucleus and cytoplasm is unknown. However, because the SCF complex is too large to diffuse passively through nuclear pores, nuclear localization might be an active process.

Multiple genetic isoforms of Skp1 are encoded in many eukaryotes (West *et al.*, 1997). Additional structural variation of Skp1 has been seen in *Dictyostelium* (in which it was previously referred to as FP21), where it is modified at HyPro143 by a linear pentasaccharide with the sequence Galα1,6Galα1,Fucα1,2Galβ1,3GlcNAc (Teng-umnuay *et al.*, 1998). The Pro residue to which the pentasaccharide is attached after hydroxylation is highly conserved in many Skp1 genes detected in fungi, invertebrates, and higher plants. Structural variants of Skp1 may differentially localize in the cell or enter into specific multiprotein complexes.

To investigate the significance of Skp1 structural variation, we examined the expression, glycosylation, and cellular localization of the two genetic isoforms of *Dictyostelium* Skp1, Skp1A and Skp1B. This has revealed that both Skp1s are stable and expressed at the message level throughout the life cycle. They

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are similarly and mostly glycosylated, by a mechanism that shows some developmental regulation and is not necessarily cotranslational. They are each enriched in the nucleus and regions of the cytoplasm, and nuclear enrichment appears to depend on its glycosylation. These results provide the first clue about the function of the unusual glycosylation of Skp1, which is mediated by a novel glycosylation pathway that appears to reside in the cytoplasm rather than the secretory pathway of the cell (West *et al.*, 1996; Teng-umnuay *et al.*, 1999).

Results

Skp1A and Skp1B are each expressed throughout the life cycle

Analysis of Skp1 is complicated by the fact that it consists of two different proteins that differ by only a single amino acid, A39 in Skp1A and S39 in Skp1B (West *et al.*, 1997). This variation is conserved among Skp1 genes in *Drosophila* (GenBank accession numbers AF220066 and AF220067). First we asked whether different Skp1s are expressed in specific cell types, or expressed coordinately throughout the life cycle. A reverse-transcription polymerase chain reaction (RT-PCR) approach was used because the proteins are so similar.

The primer for reverse transcription of total cell RNA hybridized to a region near the 3′ end of the coding region that was identical between Skp1A and Skp1B. Subsequent PCR was based on this and an upstream primer corresponding to a second shared sequence near the 5′ end of the coding region. This yielded a predominant band of about 420 bp from HL-5 grown Ax3 cells (Figure 1, lane 16), similar to the 428-bp length expected for Skp1 cDNA. No bands were detected in the absence of RNA (data not shown). A control lacking reverse transcriptase produced very faint bands at 420 and 580 bp (data not shown) attributable to Skp1A and Skp1B DNA, because Skp1B DNA contains a 154-bp intron not present in Skp1A (West *et al.*, 1997).

Restriction enzyme digestions confirmed that the amplified 428-bp band derived from Skp1A and Skp1B RNA. BclI, which cuts at nt 320 of only the Skp1A gene fragment, gave bands corresponding to 320 and 108 bp and the full-length Skp1B cDNA (data not shown). SspI, which cuts only Skp1B DNA at position 361, produced the expected fragments (361 and 67 bp) and intact Skp1A (data not shown). EcoRV, which cuts both Skp1A (at bp 193 and 274) and Skp1B (at bp 52 and 193), yielded all of the expected fragments on digestion (Skp1A: 193, 154, 81 bp; Skp1B: 235, 141, 52 bp) while leaving little DNA in the region of the original 428 bp band (Figure 1, lane 12). The residual DNA remaining at this position is probably a contaminating DNA product of the original PCR amplification, but, even if it consists of undigested Skp1 cDNA, it is present at such a low level that it does not affect the interpretation based on analysis of the digestion products. Relative ratios of the Skp1A- and Skp1B-derived bands were not affected by the number of amplification cycles from 15–35 (data not shown). EcoRV bands derived from Skp1A and

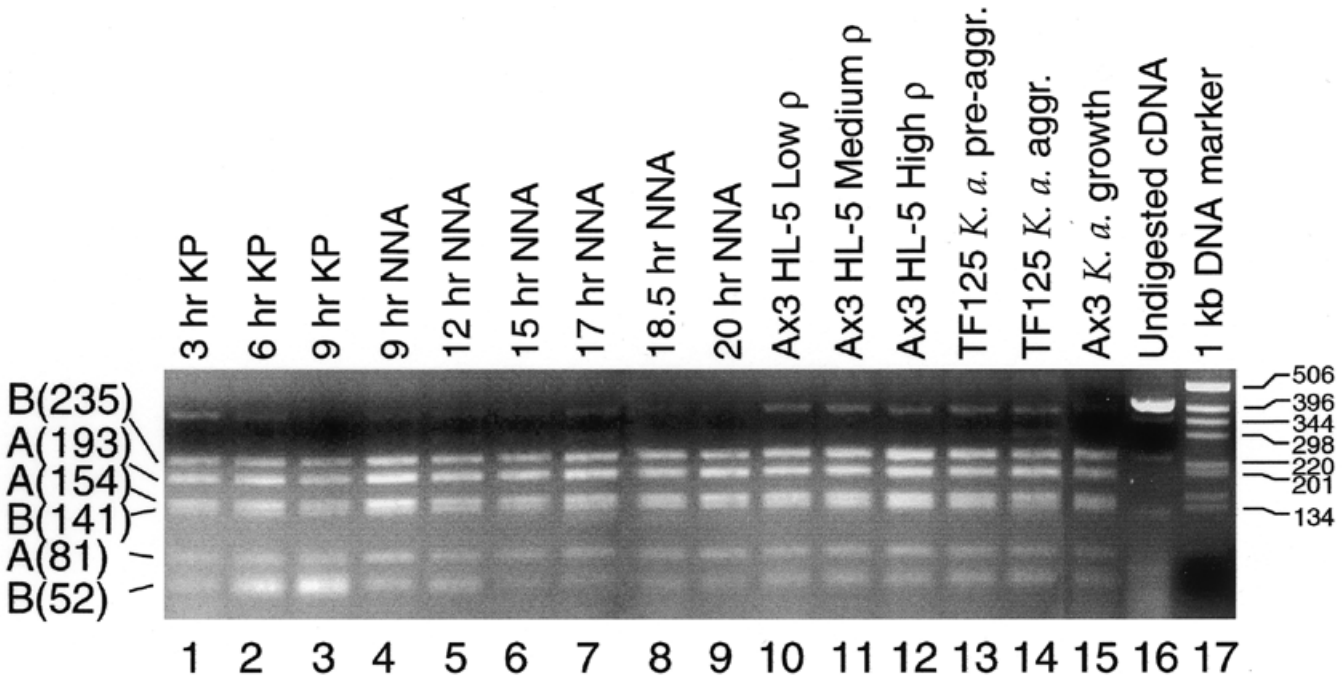

Fig. 1. RT-PCR analysis of the expression of Skp1A and Skp1B mRNAs. Normal strain Ax3 cells were taken from HL-5 growth medium at 1×10^6, 5×10^6, and 2×10^7 cells/ml (lanes 10–12). For development, HL-5 grown cells were suspended in KP buffer at 2×10^7 cells/ml and incubated on a shaker for 3, 6, or 9 h (lanes 1–3). Alternatively, cells were plated on nonnutrient agar (NNA) for 9, 12, 15, 17, 18.5, and 20 h (lanes 4–9). Cells were also grown on *K. aerogenes* and harvested before (lane 15), during (lane 13), or after (lane 14) consumption of all the bacteria, corresponding to growing and aggregating cells. In lanes 13 and 14, strain TF125, a REMI-mutagenesis strain containing a spontaneous insertion of *pyr5,6* 900 bp upstream of the transcription start site of the Skp1A gene, was examined. Total RNA was isolated from cells, and cDNA was amplified by RT-PCR using primer sequencs that were identical between the Skp1A (*fpa1*) and Skp1B (*fpa2*) genes. The cDNA was digested with EcoRV and visualized on a 3% agarose gel with ethidium bromide. Band assignments with their predicted sizes (in bp) are listed on the left. Undigested cDNA from high density HL-5 grown Ax3 cells is in lane 16, and M_r standards are in lane 17.

Skp1B had similar intensities, indicating that Skp1A and Skp1B message levels in the original RNA pool were comparable.

Similar levels of Skp1A and Skp1B cDNA were amplified by RT-PCR at all stages of the life cycle tested, including cells grown on bacteria or at different densities in HL-5 growth medium and after various times of starvation either in suspension or on filters (Figure 1). Total Skp1 mRNA levels were also similar throughout the life cycle, except that cells from HL-5 exhibited slightly higher levels (data not shown). Both Skp1A and Skp1B mRNAs are expressed at the protein level at least in stationary phase cells (saturation density in HL-5 growth medium), based on amino acid sequencing of total Skp1 from these cells (West *et al.*, 1997).

Skp1 glycosylation is affected by inhibitors of prolyl hydroxylase

Prolyl hydroxylases that modify collagen and other secretory proteins can be inhibited *in vivo* by α,α′-dipyridyl and ethyl 3,4-dihydroxybenzoate (Kivirikko and Pihlajaniemi, 1998). Because the Skp1 glycan is attached via a HyPro residue, inhibition of hydroxylation of this Pro residue would be expected to block its glycosylation. Preliminary results suggested that 1 mM α,α′-dipyridyl partially inhibits Skp1 prolyl hydroxylase activity in cell extracts (West, unpublished data). An effect of α,α′-dipyridyl on Skp1 glycosylation in cells was tested by M_r analysis of Skp1 by SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting, as affected Skp1 was expected to be reduced in mass by 851 Da. Strain Ax3 cells attached to tissue culture plastic wells were starved in KP buffer (17 mM KPO_4, pH 6.5; developmental conditions) in 0.02–1 mM α,α′-dipyridyl for 17 h. The entire cell pellet was solubilized in SDS–sample buffer and subjected to SDS–PAGE/Western blotting with mAb 3F9 (Kozarov *et al.*, 1995), using 15–20% polyacrylamide gradient gels to maximize separation of normal and nonhydroxylated Skp1. mAb 3F9 recognizes nonglycosylated Skp1 produced recombinantly in *Escherichia coli*, indicating that its epitope does not require glycosylation. After incubation in 0.05 mM α,α′-dipyridyl, the majority of Skp1 exhibited a lower lower M_r consistent with the absence of hydroxylation and glycosylation of Pro143 (Figure 2A, upper). The overall level of Skp1 was not greatly altered. At this concentration, incorporation of [^{35}S]Met and [^{3}H]Fuc into total acid precipitable material was reduced by 2% and 9%, suggesting that the effect was not the result of inhibiting overall protein synthesis via an effect on deoxyhypusyl hydroxylase (McCaffrey *et al.*, 1995). A similar effect of α,α′-dipyridyl on Skp1 M_r was seen when cells were incubated in HL-5 growth medium, where incorporation of [^{35}S]Met and [^{3}H]Fuc into total acid precipitable material was reduced by 33% and 39%. A smaller fraction of Skp1 was shifted to the lower M_r position at lower concentrations of α,α′-dipyridyl or shorter time periods of treatment (data not shown). The fraction of total unglycosylated Skp1 did not increase at higher concentrations, and cells tended to detach from the tissue culture plate. Similar partial effects on Skp1 glycosylation were observed using 200–400 μM ethyl 3,4-dihydroxybenzoate (data not shown), an inhibitor that after intracellular de-esterification is thought to function by a mechanism distinct from that of α,α′-dipyridyl (Sasaki *et al.*, 1987; Kivirikko and Pihlajaniemi, 1998). The concentrations of α,α′-dipyridyl and ethyl 3,4-dihydroxybenzoate that were inhibitory were similar to or less than those which affect collagen hydroxylation in mammalian cells (Sasaki *et al.*, 1987; Eleftheriades *et al.*, 1995). These results suggest that hydroxylation of Skp1′s Pro143 is partially sensitive to inhibitors of known prolyl hydroxylases *in vivo*. Since negligible levels of normal Skp1 were seen at the inhibited position, nearly all Skp1 is usually glycosylated in the cell.

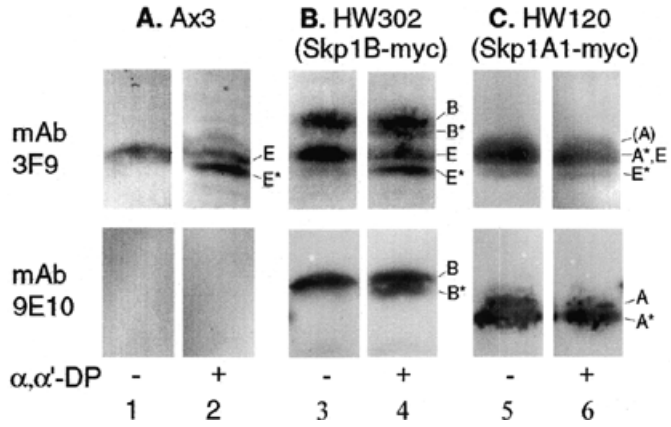

Fig. 2. M_r analysis of Skp1 from cells treated with the prolyl hydroxylase inhibitor α,α′-dipyridyl. 10^6 cells of strain (**A**) Ax3, (**B**) HW302, or (**C**) HW120, incubated in KP buffer for 17 h in the absence or presence of 50 μM α,α′-dipyridyl as indicated, were subjected to SDS–PAGE on a 15–20% gradient gel, Western blotted, and probed with either mAb 3F9 (upper panels), which recognizes both endogenous and expressed Skp1s, or mAb 9E10 (lower panels), which recognizes the C-terminal c-myc epitope tag on expressed Skp1 only. Bands marked with an E are endogenous Skp1 labeled only with mAb 3F9; bands marked with a B or A are expressed variants of Skp1B-myc or mutant Skp1A1-myc detected with both mAbs. Skp1B-myc has a lower mobility owing to the c-myc tag, which adds a predicted 1185 Da to its mass. Asterisked bands are unglycosylated variants that appear as the result of treatment with α,α′-dipyridyl; these are calculated to be 851 Da less in mass. The granular appearance of some bands results from reducing the amount of protein loaded on the gel to close to the detection threshold, to maximize band sharpness and separation.

Expressed Skp1A-myc and Skp1B-myc are similarly glycosylated at HyPro143

To investigate any differences between Skp1A and Skp1B at the protein level, it was necessary, owing to their high degree of similarity, to express them separately with epitope tags. Skp1A-myc and Skp1B-myc were initially expressed under the discoidin 1γ promoter with a c-myc tag located one residue in from their C-termini (see Table I for list of expression strains). Although the endogenous discoidin 1γ promoter is regulated *in vivo* (Blusch *et al.*, 1992), it was active in these strains throughout growth and early development (data not shown). To determine whether the myc-tagged proteins were glycosylated, their M_r values were compared in constructs in which Pro143 was substituted with Ala, as occurs in certain Skp1 gene isoforms present in *Caenorhabditis elegans* (Z81084) and *Arabidopsis thaliana* (AL138658). Whole-cell pellets of colonies expressing high levels of Skp1-myc were analyzed by SDS–PAGE followed by Western blotting with mAb 9E10, specific for the c-myc epitope on expressed Skp1. Skp1A3(P143A)-myc and Skp1B1(P143A)-myc were expressed at slightly lower M_r values than normal Skp1A-myc and Skp1B-myc (Figure 3A, compare lanes 3 and 5 to lanes 2 and

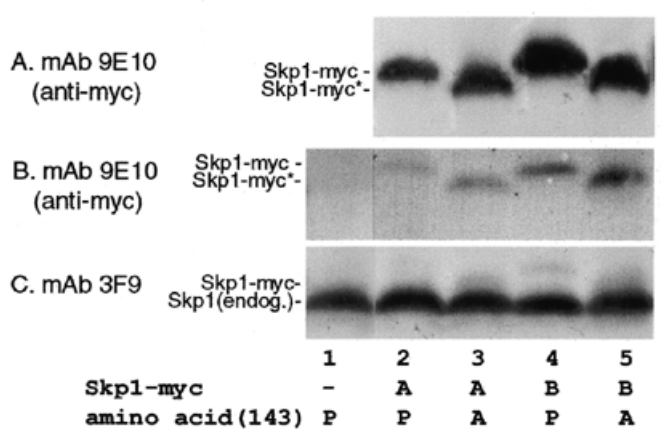

Fig. 3. M_r analysis of Skp1-myc mutants expressed under the discoidin promoter. The normal strain Ax3 (lane 1) was transfected with cDNAs encoding Skp1-myc under the control of the discoidin 1γ promoter. Lane 2, normal Skp1A-myc; lane 3, Skp1A3(P143A)-myc; lane 4, Skp1B-myc; lane 5; Skp1B1(P143A)-myc. Stationary phase (at maximum cell density in growth medium) cells were collected and analyzed as in Figure 2 by Western blotting with anti-Skp1 antibodies. (**A**) 3×10^6 growth-phase cells from high-level expression level colonies were probed with mAb 9E10 against the c-myc tag on expressed Skp1. (**B**) 10^6 growth-phase cells from low-level expression clones (listed in Table I) were probed with mAb 9E10. (**C**) A parallel blot of low-level expression strains was probed with mAb 3F9, which recognizes both endogenous and expressed Skp1. Positions of Skp1 isoforms are identified at the left; asterisks refer to nonglycosylated variants.

4), comparable to the change induced by α,α′-dipyridyl (Figure 2A). A similar M_r difference (Figure 3B) was seen in low-expressing clones employed for some of the localization studies described below. Skp1-myc was expressed in these clones at substantially lower levels than endogenous Skp1, based on comparisons of their levels using mAb 3F9 (Figure 3C). Expressed and endogenous Skp1s were readily differentiated by the increase in M_r of 1185 resulting from the c-myc epitope. To verify that normal Skp1-myc was glycosylated, strain HW302, which expresses a high level of Skp1B-myc, was treated with α,α′-dipyridyl as described above. As seen for endogenous Skp1, a fraction of Skp1B-myc migrated more rapidly in the gel (Figure 2B) to a position similar to that of Skp1B1(P143A)-myc. Altogether, these results showed that it is possible to stably express Skp1A and Skp1B with C-terminal c-myc tags at high and low levels relative to endogenous Skp1. As seen for total Skp1, both expressed Skp1A and Skp1B are nearly completely glycosylated even when expressed at high levels. In addition, substitution of Pro143 with an Ala residue blocks glycosylation, as expected, and nonglycosylated forms of the proteins are also stably expressed.

A previous study (Teng-umnuay *et al.*, 1998) examined the glycosylation of a mutant version of Skp1A1-myc produced by strain HW120 that contained two missense mutations distant from the glycosylation site (Table I). MS analysis of attachment-site peptides (139–155) showed substantial levels of peptides lacking Pro143 hydroxylation or containing this modification but lacking glycosylation, in addition to those containing partial and complete pentasaccharides. Quantitative sugar composition analysis suggested that only about 15% of Skp1A1-myc was glycosylated (Teng-umnuay *et al.*, 1998). To confirm this conclusion, Skp1A1-myc was examined by SDS–PAGE/Western blotting as above. Skp1A1-myc predominantly migrated at a single M_r position similar to that of native Skp1 (Figure 2C), not at the higher positions of Skp1A-myc with or without the P143A substitution. The reason for anomolous migration is not understood. The mobility of the main part of this band was not shifted by treatment of the cells with α,α′-dipyridyl. However, labeling of the upper region of this band appeared to be reduced, consistent with the presence of a small proportion of glycosylated protein seen in the MS and sugar composition studies. A second Skp1 mutant with point mutations in its N-terminal half, Skp1A2-myc (Table I), exhibited electrophoretic behavior that was indistinguishable from that of Skp1A1-myc (data not shown). Thus, mutant Skp1A1-myc and probably Skp1A2-myc consists predominantly of the nonglycosylated form, consistent with demonstration of the former as a substrate for the Skp1 GlcNAc-Tase (Teng-umnuay *et al.*, 1999). However, its low level of glycosylation does not appear to be the result of overexpression (compare Figure 2C with 2B) as originally surmised, but due to the missense mutations, which also cause (possibly indirectly) anomolous gel migration.

To determine whether Skp1B is glycosylated in the same way as are Skp1A and total Skp1, which were analyzed previously (Teng-umnuay *et al.*, 1998), Skp1B-myc was purified to homogeneity from the high-level expressor strain HW302, reduced, alkylated, and digested with endo-Lys-C. The resulting peptides were separated on a reverse-phase high-performance liquid chromatography (RP-HPLC) column and analyzed by matrix-assisted laser desorption ionization–time of flight–mass spectrometry (MALDI-TOF-MS). Two sequential absorbance peaks were found to contain abundant ions with *m/z* ratios of 2487 and 1636, corresponding to the MH^+ values of the pentasaccharide-peptide139–155 and peptide139–155 detected previously in Skp1A-myc (Teng-umnuay *et al.*, 1998). In-source fragmentation of the *m/z* 2487 ion revealed a series of daughter ions corresponding precisely to the sequence Hex-Hex-deoxyHex-Hex-HexNAc-HyPro-peptide, suggesting that the Skp1B oligosaccharide was identical to that of the Skp1A-myc oligosaccharide. The presence of the unmodified peptide, as indicated by the *m/z* 1636 ion, suggested the existence of unmodified Skp1B-myc in the cell, but the level of this isoform was low based on SDS–PAGE/Western blot analysis as described above (Figure 2B).

Skp1 expressed in prespore cells is not glycosylated

To investigate possible developmental regulation of glycosylation, Skp1B-myc and Skp1B1(P143A)-myc were expressed under the control of the *cotB* promoter, which is absolutely specific for prespore cells (Fosnaugh and Loomis, 1993), which appear later in development. After 20 h of starvation, at which time about 75% of the cells have differentiated into prespore cells, Western blot analysis showed that each of these Skp1s was expressed at a high level compared to endogenous Skp1 (Figure 4B, lanes 2, 3). Their apparent M_r values were indistinguishable (Figure 4A, lanes 1–3), indicating that Skp1B-myc was not glycosylated. Although the capacity of the glycosylation pathway might have been saturated by the high level of Skp1B-myc expressed, there was no evidence for even partial glycosylation of expressed Skp1.

Table I. Strains used in this report

Name	Skp1 protein expressed[a]	Promoter	Expression level[b]	% Glycosylation[c]
Ax3(A3)	parental[a]	self	normal	> 90%
HW348	Skp1A-myc	discoidin	low	> 90%
HW120	Skp1A1(I34T,D71G)-myc	discoidin	high	20%
HW351	Skp1A2(K53A,V54S,L55S)-myc	discoidin	high	20%
HW349	Skp1A3(P143A)-myc	discoidin	low	none
HW350	Skp1B-myc	discoidin	low	> 90%
HW302	Skp1B-myc	discoidin	high	> 90%
HW352	Skp1B1(P143A)-myc	discoidin	low	none
HW358	Skp1B-myc	*cotB*	high	< 10%
HW360	Skp1B1(P143A)-myc	*cotB*	high	none
TF125		self[d]	normal	> 90%
HL250		self	normal	> 90% (core disaccharide)

[a]All strains express endogenous Skp1A and Skp1B (previously referred to as FP21), encoded by the *fpa1* and *fpa2* genes. Expressed Skp1s contained C-terminal c-myc epitope tags.
[b]Low level is equivalent to < 25% of the level of endogenous Skp1 (normal); high level refers to 1–3× the level of endogenous Skp1. Levels were estimated from the ratio of cell extracts required to produce similar signals on the same Western blot.
[c]Approximate percent glycosylation was estimated from the ratio of cell extracts required to produce similar signals from the lower mobility glycosylated form and the higher mobility aglycoform on the same Western blot. An inequality means that an equivalence was not achieved at the indicated ratio. Glycosylated Skp1 appeared to express the full pentasaccharide chain based on SDS–PAGE and mass spectrometry, except for strain HL250, which expressed only the core disaccharide.
[d] Strain TF125 (provided by W.F. Loomis) contains an insertion of the *pyr5,6* gene 900 bp upstream of the *fpa1* start codon.

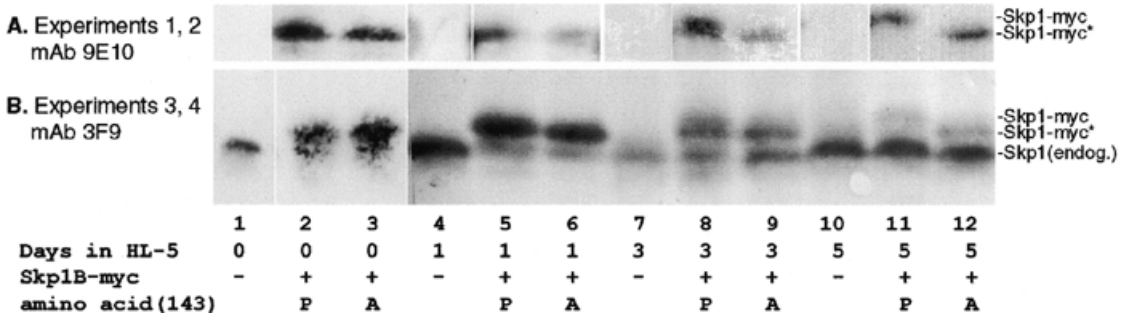

Fig. 4. M_r analysis of Skp1-myc mutants expressed under the prespore cell-specific *cotB* promoter. Strain Ax3 and clonal cell lines expressing Skp1B-myc or Skp1B1(P143A)-myc (as indicated in bottom legend) under the control of the *cotB* promoter were developed to the slug stage (18–20 h). At this time (day 0), slugs were dissociated into single cells and returned to HL-5 growth medium, which blocks *cotB* promoter activity. Whole cells were analyzed after 0 (immediately), 1, 3, and 5 days in this medium, as described above. Cell proliferation did not initiate until day 2. (**A**) Gel lanes were loaded to yield similar signal levels for Skp1B-myc and Skp1B1(P143A)-myc from different days to facilitate M_r comparisons, and the blot was probed with mAb 9E10. The day 0 results are from experiment 1 (same blot), and the day 1−5 results are from experiment 2. (**B**) Gel lanes were loaded with similar cell numbers (except lane 4, which is overloaded), to show changes in absolute levels of expression, and probed with mAb 3F9 to compare levels of expressed and endogenous Skp1. Day 0 results are from experiment 3, and the day 1–5 results are from experiment 4. Positions of Skp1 isoforms are identified at the right; asterisks refer to nonglycosylated variants. Immunoreactive material that accumulated at higher M_r positions in lanes 4–9 is not shown.

To ask if Skp1B-myc expressed in prespore cells was competent to be glycosylated, the 20-hour cells were dissociated and restored to growth medium (HL-5) to determine whether previously synthesized Skp1B-myc could be shifted up in M_r as cells began to proliferate again. After 1 day in HL-5, Skp1B-myc and Skp1B1(P143A)-myc persisted at high levels relative to endogenous Skp1 as determined by probing with mAb 3F9 (Figure 4B, lanes 5, 6), and their apparent M_r values remained indistinguishable based on probing with either mAb 3F9 or 9E10 (Figure 4A,B, lanes 5, 6). Subsequently, the levels of Skp1B-myc and Skp1B1(P143A)-myc declined and eventually became undetectable by day 6, after about five population doublings. By day 3, a higher M_r form of Skp1B-myc appeared (Figure 4A,B, lanes 8, 9), and this became the predominant isoform by day 5 (lanes 11, 12). Because the M_r of Skp1B1(P143A)-myc did not change, this increase must have

been due to subsequent glycosylation at position 143. Thus expressed Skp1B-myc was stable during this developmental transition, whether or not it was glycosylated. Because Skp1B-myc produced by prespore cells was glycosylated when cells were returned to growth phase, this suggested that its failure to be glycosylated in prespore cells was the result of down-regulation of the hydroxylation/glycosylation pathway in prespore cells, rather than an incompetence of Skp1 to be posttranslationally modified.

Localization of Skp1 in the cell

Previous studies concluded that Skp1 is concentrated in the nucleus and the centrosome, relative to the cytoplasm, based on indirect immunofluorescence microscopy of cultured mammalian cells (Freed *et al.*, 1999; Gstaiger *et al.*, 1999). Localization of Skp1 in *Dictyostelium* was investigated based on a similar approach using the mAbs described in the Western blot studies above. Growing cells of the normal strain Ax3 were allowed to attach to cover slips in HL-5 growth medium, fixed in 4% paraformaldehyde, permeabilized with cold acetone, and immunoprobed using an indirect technique with mAb 3F9. This antibody was induced against denatured, gel-purified Skp1 and is monospecific for Skp1 based on Western blot analysis (Figure 5A, lane 1), and immunoprecipitation of either native or denatured cell extracts with mAb 3F9 failed to enrich for any additional reactive bands (Larner and West, unpublished data). Fluorescence was found throughout most of the cytoplasm and was concentrated in a few patches in the cytoplasm and in the nucleus, as determined by colabeling with 4′,6′-diamidino-2-phenylindole (DAPI) (Figure 5B, panels 1–3). Only an indistinct haze was seen in the absence of primary antibody (panel 4). Cells that had become multinucleated while grown in suspension were labeled in all nuclei. Deconvolution microscopy demonstrated that Skp1 was localized in the nuclear interior (panels 1–3, insets). Similar results were obtained using mAb 4E1, an independent anti-Skp1 mAb that is also monospecific for Skp1 based on Western blot analysis (Kozarov *et al.*, 1995) (data not shown). Nuclear enrichment was also observed when paraformaldehyde-fixed cells were permeabilized with 0.1% NP-40 in place of acetone or MeOH, but was not as pronounced when cells were fixed with only cold MeOH or MeOH/HAc (data not shown), as previously reported for mammalian Skp1 (Freed *et al.*, 1999).

To determine if expressed Skp1B-myc was similarly localized, growing cells of strain HW302 were immunoprobed with mAb 9E10, which recognizes a continuous epitope associated with the C-terminal c-myc tag. mAb 9E10 is monospecific for expressed Skp1B-myc based on Western blot analysis of whole cells (Figure 5A, lanes 4, 3). Fluorescence labeling was diffuse throughout the cytoplasm and was concentrated in the nucleus (Figure 5B, panels 5–7). Only a low level of indistinct fluoresence was seen in cells of the parental strain Ax3 labeled

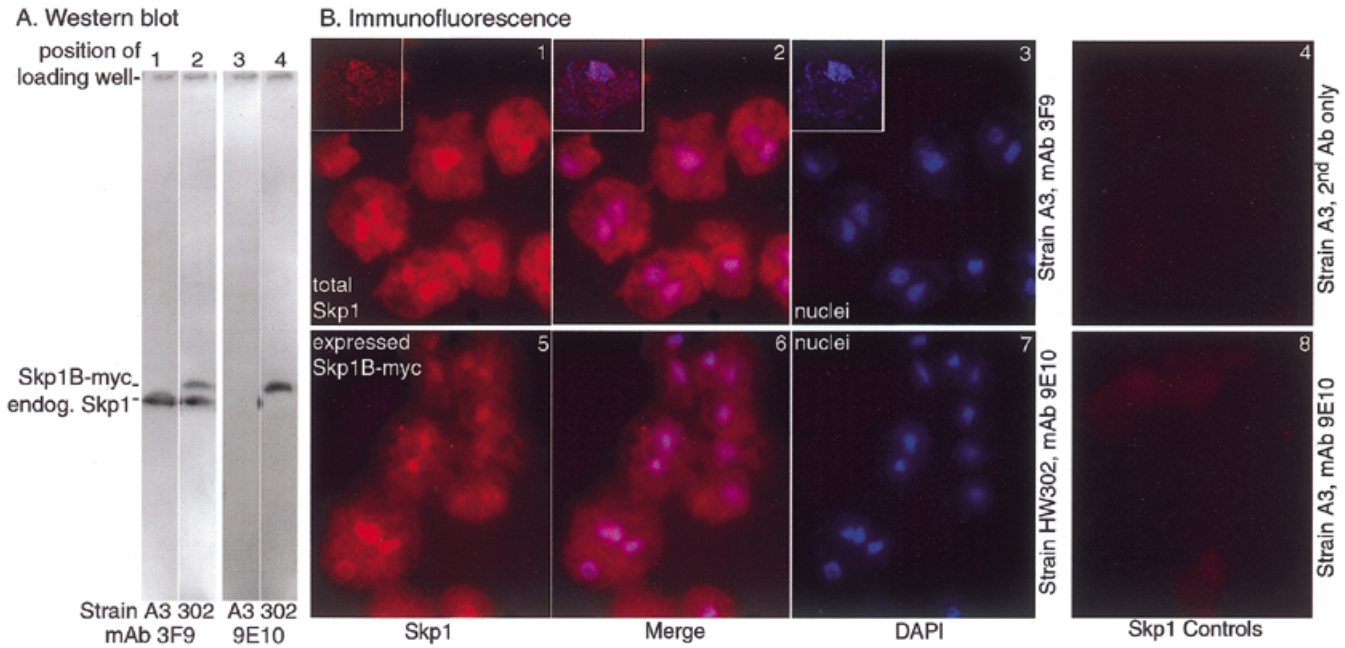

Fig. 5. Immunofluorescence localization of total and expressed Skp1 in growing cells. (**A**) The specificity of mAb 3F9 for Skp1 (lanes 1, 2), and of mAb 9E10 (lanes 3, 4) for expressed Skp1, was evaluated by Western blot analysis after SDS–PAGE on a discontinuous, 7–20% gradient polyacrylamide gel. The full length of the blot is shown. 2×10^6 cells of strain Ax3 (lanes 1, 3) or strain HW302 (lanes 2, 4) was loaded on each lane, and probing was at a 1:100 dilution of primary antibody. (**B**) Growth phase cells were allowed to attach to glass cover slips in HL-5 and fixed in paraformaldehyde and acetone. Normal strain Ax3 cells were probed with mAb 3F9 to localize total Skp1 (panel 1), and counterstained with DAPI to show nuclei and mitochondria (panel 3). Panels 1 and 3 are overlaid in panel 2. Insets: A single optical section derived from an image taken on a deconvolution microscope. Panel 4 shows strain Ax3 cells probed with the second Ab only and photographed using the same exposure conditions as panel 1. Strain HW302 cells were probed with mAb 9E10 to selectively detect expressed Skp1B-myc (panel 5) and counterstained with DAPI (panel 7); the overlay of panels 5 and 7 is in panel 6. Panel 8 shows strain Ax3 cells probed with mAb 9E10, photographed using the same exposure conditions as panel 5.

with mAb 9E10 (panel 8), showing that fluorescence localization was specific for expressed Skp1B-myc. Skp1A-myc was similarly localized in strain HW348 (see Figure 7A,B below). The congruent nuclear concentration of endogenous Skp1 detected by mAbs 3F9 and 4E1 and expressed Skp1A-myc and Skp1B-myc detected by mAb 9E10 strongly supports the interpretation that Skp1 is normally enriched in this organelle. This interpretation is in agreement with similar conclusions drawn from different antibodies that Skp1 is nuclear-enriched in mammalian cell lines (Freed *et al.*, 1999; Gstaiger *et al.*, 1999).

In an attempt to gain biochemical evidence for nuclear enrichment of Skp1, previous experiments that failed to detect Skp1 in purified nuclei (Kozarov *et al.*, 1995) were repeated with similar negative results (data not shown). Inefficient recovery of Skp1 after purification of mammalian cell nuclei was previously reported (Gstaiger *et al.*, 1999). This suggests that Skp1 belongs to the major class of nonbound nuclear proteins that are rapidly lost from nuclei isolated in aqueous buffers (Allfrey, 1959; Paine *et al.*, 1983), which is consistent with the lower level of Skp1 seen in alcohol-fixed compared to aldehyde-fixed cells (see above).

The localization of Skp1 in early developing cells preparing for chemotactic aggregation was investigated after transferring cells into KP buffer for 2–8 h. The nuclear concentration of endogenous and expressed Skp1 that was seen in growing cells was conserved, but cytoplasmic labeling along the plasma membrane and in cytoplasmic extensions was more prevalent (Figure 6A–D). Cytoplasmic labeling was often (but not always) correlated with F-actin as determined by colabeling with FITC-phalloidin. Prespore cells, formed late in development, also showed nuclear enrichment of Skp1 (data not shown). The varied range of distributions suggested that Skp1's cytoplasmic localization is dynamic, but that its enrichment in the nucleus is constant.

Nuclear enrichment of Skp1 requires glycosylation

The potential role of glycosylation in Skp1 localization was examined in the low-level Skp1-myc expression strains described above in which the glycosylation site had been mutated from P to A. When expressed in growing cells under the discoidin promoter, no nuclear enrichment of Skp1A3(P143A)-myc was observed in any of the hundreds of cells examined in multiple trials (Figure 7C,D). In contrast, total Skp1 was concentrated in the nucleus in the same population of cells (Figure 7E), and normal Skp1A-myc was concentrated in the nucleus (Figure 7A,B). Thus the P143A substitution, which occurs naturally in some metazoan Skp1 genes, appeared to block both glycosylation and nuclear concentration.

Two Skp1A-myc mutants described above, Skp1A1-myc and Skp1A2-myc (Table I), are poorly glycosylated and thus provided an opportunity to explore the relationship between glycosylation and nuclear concentration further. Skp1A1-myc was not enriched in the nucleus, but its localization in the cytoplasm of cells developed in KP buffer appeared normal (Figure 6E,F). Skp1A2-myc was similarly not concentrated in the nucleus (data not shown). These results show that when glycosylation is blocked by a different mechanism than the P143A mutation, nuclear concentration relative to the cytoplasm still does not occur. Because most Skp1A1-myc is

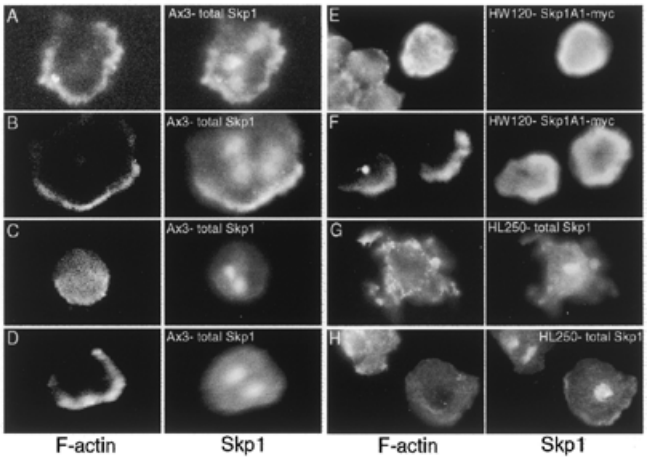

Fig. 6. Localization of total and expressed Skp1 in developing cells. Growth phase cells were incubated in KP buffer for 8 h on cover slips, fixed, and immunolabeled as shown in Figure 5 except that cells were counterstained with FITC-phalloidin to localize F-actin. (**A**–**D**) Four examples of normal strain Ax3 labeled with mAb 3F9, showing the variable subplasmalemmal localization of Skp1 that in some cases overlaps with F-actin. (**E**, **F**) Two examples of the localization of expressed mutant Skp1A1-myc in strain HW120 using mAb 9E10. (**G**, **H**) Two examples of the localization of Skp1 in strain HL250 using mAb 3F9, in which Skp1 is modified by only the core disaccharide. The mAb-dependent focal cytoplasmic labeling in panels A–D,G and H correspond to nuclei.

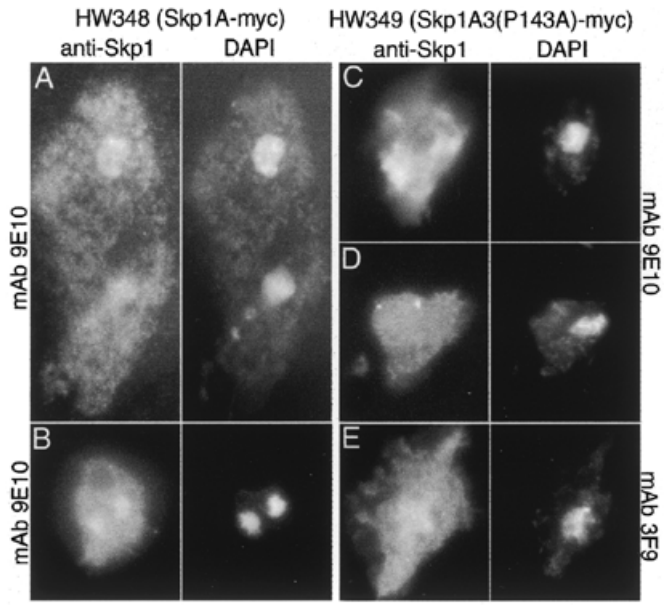

Fig. 7. Localization of the P143A mutant of Skp1A in growing cells. Growth phase cells were attached to cover slips and imunoprobed as in Figure 5. (**A**, **B**) Strain HW348 cells were probed with mAb 9E10 to localize expressed Skp1A-myc and DAPI to localize nuclei. (**C**, **D**) Strain HW349 cells were probed with mAb 9E10 to localize expressed Skp1A3(P143A)-myc, which is not glycosylated, and DAPI, to localize nuclei. (**E**) Strain HW349 cells were probed with mAb 3F9 to localize total Skp1.

hydroxylated at Pro143 (Teng-umnuay *et al.*, 1998), hydroxylation alone was not sufficient for nuclear enrichment relative to the cytoplasm.

Localization of Skp1B-myc expressed in prespore cells, where it was not glycosylated, was also found to be not enriched in the nucleus (Figure 8A). This result was confirmed by deconvolution microscopy (Figure 8B). Its localization was similar to expressed Skp1B1(P143A)-myc (Figure 8C). Endogenous Skp1 was still nuclear-enriched in these cells (data not shown), showing they had not lost capacity of nuclear concentration. Nucleoli were especially devoid of Skp1B-myc (Figure 8C), and in some cells, Skp1 appeared to accumulate at the nuclear surface (Figure 8C).

Finally, the localization of total Skp1 in cells whose glycosylation was partially inhibited using prolyl hydroxylase inhibitors was examined. Both α,α′-dipyridyl and ethyl 3,4-dihydroxybenzoate (DHB), at concentrations found to inhibit Skp1 glycosylation, appeared to reduce the nuclear concentration of Skp1. Shown in Figure 9 is an example from a concentration series of DHB applied to HW302 cells in KP buffer for 17 h. At 0.2 mM DHB, the fluorescence intensity of mAb 3F9 labeling of nuclei relative to cytoplasm was slightly reduced relative to the untreated control (compare Figure 9B and A). This effect was more pronounced at 0.4 mM and 0.6 mM DHB, but a slight enrichment of Skp1 in nuclei relative to adjacent cytoplasm was observed even at the highest concentration. This was consistent with an inability of the inhibitors to block all Skp1 hydroxylation (Figure 2). The effect of DHB was also assessed by counting the number of DAPI-positive nuclei that were not selectively immunolabeled relative to the surrounding cytoplasm in Photoshop images. This fraction was 0.10, 0.17, 0.44, and 0.41 for the concentration series of 0, 0.2 mM, 0.4 mM and 0.6 mM shown in Figure 9 (N = 200 per data point). These results show that when Skp1 glycosylation is inhibited by a mechanism that is independent of altering Skp1 structure, the ability of Skp1 to be concentrated in the nucleus is still compromised.

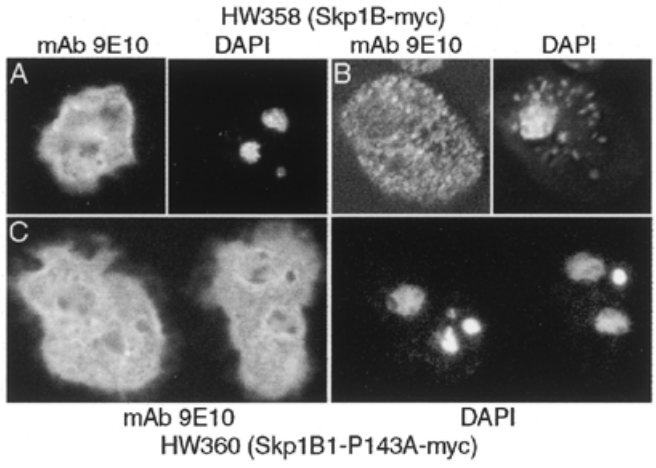

Fig. 8. Localization of Skp1B-myc expressed in prespore cells. Cells were developed to the slug stage of development, dissociated into single cells, and allowed to attach to cover slips. Fixation and immunoprobing were as in Figure 5. (**A**) Strain HW358 was immunoprobed with mAb 9E10 to localize expressed Skp1B-myc, and counterstained with DAPI to locate nuclei. (**B**) As in A, except that this is a single optical plane from a deconvolution image. (**C**) Strain HW360 was immunoprobed with mAb to localize expressed Skp1B1(P143A)-myc and counterstained with DAPI to locate nuclei. Variations in nuclear staining occur because some are out of focus. Nucleoli are evident as DAPI-negative zones in the nuclei.

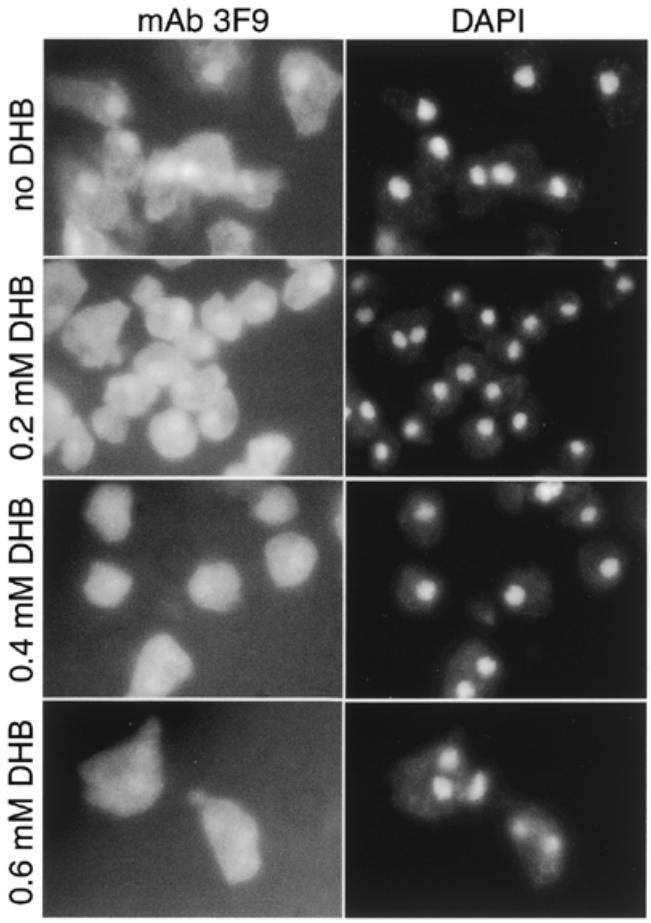

Fig. 9. Localization of Skp1 when glycosylation was inhibited by ethyl 3,4-dihydroxybenzoate. HW302 cells were incubated at the indicated concentrations of ethyl 3,4-dihydroxybenzoate in KP buffer for 17 h on cover slips. Cells were processed for immunofluorescence as described in Figure 5, using mAb 3F9 for total Skp1 (left panels) and DAPI-staining to locate nuclei (right panels).

To examine whether the entire pentasaccharide modification is important for nuclear accumulation, Skp1 was localized in a GDP-Fuc synthesis mutant (HL250). In HL250, Skp1 contains only the Galβ1,3GlcNAc core disaccharide at HyPro143 (Teng-umnuay *et al.*, 1998). Nevertheless, HL250 Skp1 exhibited a high level of nuclear concentration (Figure 6G,H), showing that only the core disaccharide is important for nuclear concentration.

Discussion

Dictyostelium Skp1 is found in both the nucleus and cytoplasm

Three independent monoclonal antibodies that are monospecific for Skp1 based on Western blot analysis were used to

localize Skp1 in cells by indirect immunofluorescence. One of the mAbs is specific for epitope-tagged Skp1, and the other two probably recognize continuous epitopes that are likely to be faithfully displayed by the Western blot method used to assess specificity. Fluorescence signals were substantially higher than control levels in the nucleus and in a heterogeneous pattern in the cytoplasm, and fluorescence signals over nuclei were substantially greater compared to adjacent cytoplasm (Figure 5). Fluorescence originated from within the nucleus based on decovolution microscopy. No differences in nuclear enrichment were observed between endogenous Skp1 and expressed Skp1A-myc or Skp1B-myc, indicating that the C-terminal c-myc tag did not interfere with Skp1 localization. Similar results were obtained when cells were fixed with paraformaldehyde or organic solvents, and thus nuclear concentration of Skp1 is unlikely to be a stress response to the aldehyde. Enrichment of Skp1 in the nucleus is consistent with similar results obtained in cultured mammalian cells (Freed *et al.*, 1999; Gstaiger *et al.*, 1999) and, as previously noted, is consistent with the compartmentalization of some target substrates of SCF complexes there.

In developing cells, substantial fluorescence was also concentrated over regions of peripheral cytoplasm either at the cell margin or cell top, which sometimes superimposed over actin-rich regions of the cell (Figure 6). Localization in the cytoplasm is consistent with the compartmentalization of other substrates of SCF complexes, and data that Skp1 interacts with the actin-binding protein coronin (Uetz *et al.*, 2000) and the barbed ends of F-actin *in vitro* (Bubb *et al*., unpublished data). Localization in the centrosome, seen previously using polyclonal antisera in mammalian cells (Freed *et al.*, 1999; Gstaiger *et al.*, 1999), was not observed in *Dictyostelium* using our mAbs. Despite its glycosylation, Skp1 is not vesicular consistent with the absence of targeting sequences or other evidence for entering the secretory pathway (Kozarov *et al.*, 1995).

Enrichment of Skp1 in the nucleus relative to the surrounding cytoplasm was consistently observed at all stages of development examined. This suggested that Skp1 might also be enriched in isolated nuclei relative to other cellular fractions, but this was not observed (Kozarov *et al.*, 1995). Loss of proteins from nuclei isolated in aqueous media has been documented (Allfrey, 1959; Paine *et al.*, 1983) and was also observed for mammalian Skp1 (Gstaiger *et al.*, 1999). However, the interpretation that Skp1 is normally concentrated in nuclei is strengthened by evidence, described below, that this apparent localization is modulated by specific structural changes in Skp1.

Nuclear concentration of Skp1 depends on its glycosylation

When the glycosylation site was mutated from Pro to Ala, Skp1A3-myc was not enriched in the nucleus relative to the nearby cytoplasm (Figure 7C,D). This did not appear to be the result of saturating the nuclear concentration process, as Skp1A3-myc was expressed at low levels in these strains relative to endogenous Skp1 (Figure 3), and high levels of expressed Skp1B-myc in other strains were still concentrated in the nucleus (Figure 5B, panels 5–8). However, mutant Skp1A3-myc did not appear to be absolutely excluded from the nucleus, though the exact level is uncertain because it was difficult to precisely establish the level of nonspecific labeling owing to variations in cell shape. It is unlikely that the glycosylation site mutation simply destabilized Skp1, as this amino acid substitution occurs naturally in certain Skp1 genes of *A. thaliana* and *C. elegans*, and Skp1B1-myc expressed in prespore cells was as stable as normal expressed Skp1B-myc (Figure 4).

A similar correlation between loss of nuclear enrichment and lack of glycosylation was also seen in mutants with amino acid substitutions at codons 34 and 71 (Skp1A1-myc), or codons 54–56 (Skp1A2-myc) (Figures 2C, 6E,F). In contrast, concentration in the peripheral cytoplasm of developing cells was unaffected (Figure 6). Nuclear enrichment was also lost when Skp1B-myc was expressed in prespore cells (Figures 4, 8), in which case the lack of glycosylation did not involve any mutations in Skp1 itself. Finally, when Skp1 glycosylation was partially inhibited by the independent method of treating cells with the prolyl hydroxylase inhibitors α,α′-dipyridyl (Figure 2A) or ethyl 3,4,-dihydroxybenzoate, the concentration of nuclear Skp1 relative to nearby cytoplasm also appeared reduced (Figure 9). Taken together, these results strongly indicate that glycosylation of Skp1 is required for its enrichment in the nucleus relative to its level in nearby cytoplasm. However, an alternative explanation might be that absence of glycosylation resulted in change in Skp1 binding in the nucleus, thereby masking both the mAb 3F9 and 9E10 epitopes in the nucleus selectively. Such a differential effect on nuclear and cytoplasmic forms of Skp1 seems unlikely, however, because the effect was seen after acetone permeabilization and because most cellular Skp1 is thought to be bound in similar SCF complexes, but ruling out this formal possibility must await future studies.

The core disaccharide was sufficient for nuclear enrichment, as Skp1 was localized normally in the nuclei of the general fucosylation-defective strain HL250. Pro143 hydroxylation itself was insufficient, as Skp1A1-myc, which is mostly hydroxylated but not glycosylated (Teng-umnuay *et al.*, 1998), was not enriched in the nucleus (Figure 6E,F).

Possible mechanism of nuclear concentration

Although the great majority of Skp1 is glycoslyated, only a fraction of it is present in the nucleus. Thus, glycosylation seems to be necessary but not sufficient for nuclear accumulation. In cell extracts, the majority of Skp1 is inaccessbile to immunoprecipiation by any of the anti-Skp1 mAbs except after denaturation, reduction, and alkylation (Larner and West, unpublished data), suggesting that *Dictyostelium* Skp1 is in protein complexes such as the SCF complex that would be too large to enter the nucleus unless actively transported. Although it is not known if complex formation occurs before or after Skp1's nuclear concentration, it is unlikely that glycosylation is necessary for SCF complex formation as recombinant Skp1 from *E. coli*, which is not glycosylated, enters the SCF complex (Deshaies, 1999). In addition, the glycosylation site in *Dictyostelium* maps to the opposite side of the contract interface between mammalian Skp1 and the F-box protein (Schulman *et al.*, 2000).

Previous studies with neoglycoproteins have suggested that sugars can potentiate nuclear uptake. When serum albumin is derivitized with 20 or more mono- or disaccharides of Glc or GlcNAc, it can be concentrated in permeabilized mammalian cells by a process that requires ATP (Duverger *et al.*, 1995,

1996). If the Galβ1,3GlcNAc-disaccharide of Skp1 is a structural mimic of neoglycosylated serum albumin on at least one site, then the nuclear enrichment of Skp1 might be related to stimulated uptake of neoglycoproteins.

The effect of Skp1 glycosylation on nuclear concentration might be mediated by a receptor. Eukaryotic cells contain abundant levels of galectins in the cytoplasm and nucleus (for example, Gaudin *et al.*, 2000), including some that recognize substituted Galβ1,3GlcNAc core disaccharides as seen in Skp1 (Henrick *et al.*, 1998). *Dictyostelium* expresses discoidins with related binding specificities at high levels in the nucleus and cytoplasm (Erdos and Whitaker, 1983; Alexander *et al.*, 1992). Thus, cytoplasmic/nuclear lectins are potential candidates for participating in the mechanism of nuclear enrichment of Skp1.

Nuclear concentration of Skp1 might involve a shuttling mechanism in which the balance of import over export (Feldherr, 1998; Kaffman and O'Shea, 1999) is influenced by glycosylation. Skp1 harbors an amino acid sequence upstream of its glycosylation site, 96-LFELILAANYL, that resembles Crm1-dependent nuclear export signals in proteins of other organisms (Nix and Beckerle, 1997). Phosphorylation can promote or inhibit nuclear accumulation of some proteins (Kaffman and O'Shea, 1999), including inhibiting StatA nuclear association in *Dictyostelium* (Ginger *et al.*, 2000). Interestingly, the Skp1 glycosylation site is adjacent to a string of four Glu residues and might cover this acidic patch. Alternatively, glycosylation may support nuclear localization indirectly via an effect on Skp1 folding or SCF-complex assembly.

Posttranslational modification of Skp1

Skp1 appeared to be constitutively expressed and glycosylated, except that when it was expressed in prespore cells under the *cotB* promoter it was not glycosylated (Figure 4). This indicates that an early step in the pathway or a critical donor substrate or cofactor is developmentally regulated. However, this effect may not be physiologically significant because it is not known whether Skp1 is normally produced by prespore cells, and nonglycosylated variants of endogenous Skp1 were not detected late in development (Figure 4). However, Skp1 expressed in these cells was stable and permitted an analysis of nuclear concentration and potential reglycosylation (see above). Both glycosylated and unglycosylated Skp1 were stable and long-lived, as they were detectable for up to 5 days when the prespore cells were returned to growth medium. Furthermore, prespore-expressed Skp1B-myc became partially glycosylated during this interval (Figure 4), showing that glycosylation is not necessarily coupled to its biosynthesis. Another conclusion that can be derived from the studies on mutant Skp1A1-myc and Skp1A2-myc is that glycosylation depends on the structure of Skp1, as their point mutations, which map to the N-terminal half of the protein distant from the glycosylation site, block its glycosylation (Table I).

The hypothetical Skp1 prolyl hydroxylase appears to be related to known prolyl hydroxylases based on the sensitivity of Skp1 glycosylation to two different inhibitors of prolyl hydroxylases. Hydroxylation is thought to occur at the 4-position of Pro143 as a synthetic peptide containing 4-OH-Pro is a substrate of the Skp1-GlcNAc-transferase (Teng-umnuay *et al.*, 1999). All known prolyl hydroxylases are residents of the lumen of the rER (Kivirikko and Pihlajaniemi, 1998), which contrasts with the localization of Skp1 in the cytoplasm and nucleus. Current evidence suggests that Skp1 receives its GlcNAc and Fuc residues in the cytoplasm (West *et al.*, 1996; Teng-umnuay *et al.*, 1999); further work is required to localize the Skp1 prolyl hydroxylase.

Inhibitors of prolyl-4-hydroxylase have received considerable interest for their potential to interfere with collagen deposition, which depends on 4-hydroxylation of its Pro residues, in such diseases as liver cirrhosis and atherosclerosis. Although there is evidence for efficacy in cirrhosis, the mechanism seems to involve inhibition of differentiation of collagen secreting cells rather than a direct effect on collagen deposition per se (Matsumura *et al.*, 1997; Sakaida *et al.*, 1999). A possible explanation for this and other effects (McCaffrey *et al.*, 1995) is action on previously unknown prolyl hydroxylases that modify cytoplasmic or nuclear rather than secretory substrates.

Summary

There is no evidence for differential expression or glycosylation of the two Skp1 proteins expressed in *Dictyostelium*. Both Skp1A and Skp1B are nearly quantitatively modified by similar if not identical pentasaccharides at Pro143, except when produced in prespore cells. Skp1 produced in this cell type is not fully glycosylated but can be subsequently modified when cells are returned to growth medium. The unusual oligosaccharide-protein linkage of Skp1 is susceptible to inhibitors of prolyl hydroxylases *in vivo*, which provides a new method for detecting this type of complex glycosylation on other cytoplasmic/nuclear proteins by M_r-shift analysis. Prevention of glycosylation either by mutation of the attachment site or other approaches inhibited concentration of Skp1 in the nucleus relative to the surrounding cytoplasm based on immunofluorescence analysis. Although the mechanism of this effect remains to be elucidated, it is apparent that this glycosyl modification has a functional consequence on the compartmentalization of this protein.

Materials and methods

Cell strains and manipulations

Strain Ax3 was grown either axenically in HL-5 growth medium on a gyratory shaker, or on SM agar plates in association with *Klebsiella aerogenes*. For development, axenically grown cells were washed in KP buffer (17 mM KPO_4, pH 6.5), and either deposited on cover slips in KP buffer, maintained in suspension at 2×10^7/ml in KP buffer or deposited on nonnutrient agar at 1.2×10^8 cells per 10-cm-diameter plate (West *et al.*, 1997). On cover slips or in suspension, cells enter the developmental phase but did not aggregate, which normally occurs after 6–9 h on nonnutrient agar surfaces. Cells develop into slugs after 16–20 h on nonnutrient agar; about 80% of the slug cells are prespore cells in which the *cotB* promoter is active (Fosnaugh and Loomis, 1993). To dissociate prespore cells, slugs were sheared through a 26-gauge needle in 20 mM EDTA, 10 mM potassium phosphate, pH 7.5. The resulting single cell suspension was washed in KP buffer by centrifugation and resuspended in HL-5 medium. Strain TF125, isolated by W. F. Loomis, contained an insertion of *pyr*5,6 about 900 bp upstream from the Skp1A transcription start site. Strain HL250 cannot synthesize GDP-Fuc from GDP-Man and is a general fucosylation-null strain (Gonzalez-Yanes *et al.*, 1989).

RT-PCR

Aliquots of 10^7 cells were washed by centrifugation with KP buffer and frozen at –80°C in 1.5-ml microcentrifuge tubes. Samples were lysed with 1 ml TRIZOL™ Reagent (Life Technologies) by repetitive pipetting and incubated for 5 min at room temperature. Chloroform (0.2 ml; Fisher HPLC grade) was added, and the tubes were shaken vigorously and incubated again for 5 min at room temperature. Tubes were centrifuged at 12,000 × *g* for 10 min at 4°C. RNA was precipitated from the upper, aqueous phase by mixing with 0.5 ml of isopropanol, incubating for 10 min at room temperature, and centrifuging at 12,000 × *g* at 4°C for 10 min. The pellet was rinsed with 1 ml of 75% ethanol and dissolved in 50 µl of water, yielding 100–150 µg of total RNA. One microgram RNA was subjected to reverse transcription using 20 pmol of the downstream primer (5′-TCTTCACACCATTCATTTTCTTTTCT), 1 mM of each dNTP, 20 U RNase inhibitor (Perkin Elmer), 5 mM $MgCl_2$, and 50 U MuLV reverse transcriptase (Perkin Elmer), in a total vol of 20 µl of 1× PCR buffer. The reaction was allowed to proceed for 15 min at 42°C, 5 min at 99°C, and 5 min at 5°C. The reaction mixture was diluted fivefold in 1× PCR buffer to a final concentration of 2 mM $MgCl_2$ and supplemented with 2.5 U AmpliTaq® DNA polymerase and 20 pmol of the upstream primer (5′-ATTGAAAAAGAAATCGCTTGTATGT). Primers corresponded to sequences that were identical in *fpa1* and *fpa2* (Skp1A and Skp1B genes). Amplification was carried out from 15 to 35 cycles (45 s, 94°C; 45 s, 49°C; 3 min, 72°C). DNA products were resolved by electrophoresis in 1% agarose and visualized with ethidium bromide. In some cases RT-PCR products were digested with SspI, BclI, and EcoRV and visualized after electrophoresis in 3% NuSieve GTG Agarose (FMC Bioproducts).

Metabolic labeling

To test the effects of prolyl hydroxylase inhibitors on general metabolism, growth phase cells were deposited at 10^7 cells/well of a 24-well tissue culture plate in HL-5 growth medium. After cell attachment (15 min), the medium was replaced with 320 µl 0–1000 µM α,α′-dipyridyl (Sigma) or 0–400 µM ethyl 3,4-dihydroxybenzoate (Sigma) prepared in HL-5. After 30 min, the medium was supplemented with [^{35}S]Met (1000 Ci/mmol; New England Nuclear) or [6-^{3}H]L-Fuc (85.2 Ci/mmol; New England Nuclear), which had been dried down and reconstituted at 10× strength in HL-5, to a final concentration of 10 µCi/ml, and cells were allowed to incubate for an additional 3 h. For labeling of differentiating cells, HL-5 was replaced with KP buffer after cell attachment, and cells were incubated for 6 h in KP buffer before drug treatment and metabolic labeling as above. Cells were transferred to 15-ml tubes, diluted in KP, and centrifuged to recover the washed cells. The pellet was resuspended in 1 ml of 0.2% NP-40 in KP buffer. An aliquot of 0.25 ml was transferred to a microcentrifuge tube and supplemented with 200 µl 0.125 mg/ml bovine serum albumin in water. Protein was precipitated with 50 µl of 100% trichloroacetic acid for 2 h on ice and collected on a GF/C glass fiber filter, rinsed with cold 10% trichloroacetic acid and acetone, and assayed by liquid scintillation counting.

Mass spectrometry of glycopeptides

Skp1B-myc was purified from strain HW302, digested with endo-Lys-C, and fractionated on a C_{18}-reversed-phase column as described previously (Teng-umnuay *et al.*, 1998). All fractions were analyzed by MALDI-TOF-MS on a Perceptive Biosystems Voyager DE in the UF Protein Chemistry Core Lab as also described in that report.

Skp1 expression constructs

The expression constructs were derived from pVEIIΔATG by insertion of C-terminal myc-tagged Skp1-coding cDNA into the KpnI and SacI sites of its MCS, positioned downstream of the discoidin 1γ promoter intended to drive expression during late growth phase and early development, as previously described (Teng-umnuay *et al.*, 1998, 1999). The original strain HW120 expressed a version of Skp1 (Skp1A1-myc) with two PCR-derived missense mutations (Table I). A plasmid expressing normal Skp1A-myc was constructed by repeating the procedure. The Skp1B-myc expression construct was created by introducing an A39S substitution in the Skp1A-myc plasmid using the QuikChange site-specific mutagenesis kit (Stratagene, La Jolla, CA), with 5′-GGTGAATCAGATGCGCCAATTCCATTA as a primer. A newly created HhaI site was used to screen for the altered plasmid. P143A substitutions were created by similar site-specific mutagenesis, using 5′CAAGAACGACTTTACTGCAGAAGAAGAAGAAC as a primer and screening for the newly created PstI site. Skp1A2-myc, which contained amino acid substitutions at codons 53–55, was similarly prepared using GCACTATTTTAGAAGCTTCTTCTGACTATTGCAGAC as a primer, and screening for the introduced HindIII site. All sequences were confirmed directly. Myc-tagged Skp1 was expressed throughout growth and early development in these strains, and the expected substantial suppression by folic acid (Blusch *et al.*, 1992) was not observed (data not shown). To direct expression of the Skp1 constructs in prespore cells, the discoidin promoter was replaced by the *cotB* promoter as previously described (Zhang *et al.*, 1999). Plasmids were transfected into *Dictyostelium* strain Ax3 as $CaPO_4$ precipitates (Blusch *et al.*, 1992) or by electroporation (Pang *et al.*, 1999), and transformants were isolated in the presence of 15 µg/ml G418 and cloned.

Electrophoresis

Cells were pelleted in a microcentrifuge tube, lysed in Laemmli sample buffer, and separated on 15–20% linear gradient SDS–polyacrylamide slab gels. Gels were electrophoretically transferred on a semi-dry apparatus and immunochemically probed as described elsewhere (West *et al.*, 1997).

Immunofluorescence localization

Growing cells from HL-5 medium were deposited on glass cover slips and allowed to attach for 15 min. In some cases, attached cells were either maintained in HL-5 or rinsed in KP buffer and allowed to develop, for up to 17 h, in the absence or presence of a range of concentrations of α,α′-dipyridyl or ethyl 3,4-dihydroxybenzoate. Cells were fixed by treatment with 2–4% paraformaldehyde in KP buffer, pH 7.4, for 15 min, followed by treatment with either acetone (–20°C) or 0.1% NP-40 in KP buffer for 5 min. Similar results were obtained with both methods. Alternatively, cells were fixed in 50%

acetone:50% methanol at room temperature for 2 min, followed by replacement with phosphate buffered saline (PBS), or in 95% ethanol:5% glacial HAc at –20°C for 5 min, followed by PBS (Harlow and Lane, 1988). After rinsing in KP or PBS, cells were blocked in 5% nonfat dry milk in TBS for 5 min, followed by incubation in 1°C antibody diluted in the milk solution for 1 h, rinsing in TBS, and incubation in 1:200 dilution of affinity-purified Texas red conjugated rabbit anti-mouse antibody (Sigma). Alternatively, fixed cells were blocked in 3% (v/v) goat serum in PBS, incubated with antibodies diluted in 1% goat serum, and probed with a 1:2,000 dilution of affinity-purified Alexa 568-conjugated goat anti-mouse antibody (Molecular Probes). In some cases, cells were subsequently incubated with FITC-phalloidin (Molecular Probes) to detect F-actin. FITC-phalloidin was prepared by drying an aliquot in a vacuum centrifuge, replacing with the same volume of 10% Triton X-100, and diluting 1200-fold in PBS. After final washing in TBS, cover slips were mounted in Vectashield (Vector Labs) containing 0.1 μg/ml DAPI. Images were collected digitally on a Zeiss Axiophot microscope equipped with a SPOT-2 camera, or on an Applied Precision deconvolution microscope equipped with Deltavision software. Multiple color channel recordings were taken from the same focal plane. Related images were processed similarly in Adobe Photoshop. Conclusions were based on multiple trials involving many cells viewed by independent observers. Unless otherwise indicated, cells shown were representative of the entire population.

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Abbreviations

DAPI, 4′,6′-diamidino-2-phenylindole; DHB, dihydroxybenzoate; KP, 17 mM KPO_4, pH 6.5; MALDI-TOF-MS, matrix-assisted laser desorption ionization–time of flight–mass spectrometry; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; RP-HPLC, reverse-phase high-performance liquid chromatography; RT-PCR, reverse-transcription polymerase chain reaction; TBS, Tris buffered saline.

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