Identification of a novel Skp2-like mammalian protein containing F-box and leucine-rich repeats[1]

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Abstract The F-box protein Skp2 is important for S phase entry and binds to Skp1 and the cyclin A-Cdk2 complex. Here we report the cloning, analysis of genomic organization and characterization of a novel gene product related to Skp2 named FBL2. The human FBL2 gene was found to be a highly interrupted gene of at least 126.6 kb located on chromosome 17 in close proximity to the TRAP220 gene in a head-to-tail orientation. The predicted protein contains an F-box and six perfect C-terminal leucine-rich repeats. Similar to Skp2, this protein interacts with Skp1 and deletion of the F-box inhibits this association. However, in contrast to Skp2, FBL2 was detected in non-proliferating hepatocytes and its expression increased in growth-arrested liver epithelial cells. In addition, FBL2 was localized primarily in the cytoplasm concentrated around the nucleus. Overall, our data indicate that although FBL2 shares strong structural homology with Skp2 as well as having a similar ability to associate with Skp1, these proteins likely play distinct roles and target different substrates to the SCF complex.

Key words: F-box; Skp1; Skp2; SCF complex

1. Introduction

The activity of cyclin-dependent kinases (Cdks), key regulators of cell cycle progression, is determined by the balance of positive regulatory subunits known as cyclins and negative factors such as Cdk[2] inhibitors. Expression of these proteins is tightly controlled at the level of transcription, translation and, finally, protein stability. One of the important post-transcription mechanism of regulating protein abundance in eukaryotes is a ubiquitin-dependent proteolytic pathway.

Genetic analyses in yeast have identified a group of genes involved in the ubiquitin-dependent proteolysis of cell cycle control proteins such as cyclin-dependent kinase inhibitors Sic1 and Far1 as well as G1 cyclins Cln1 and Cln2 (reviewed in [1–3]). Biochemical studies reveal that products of these genes form a multi-protein complex in yeast cells, the invariable core consisting of Cdc34, Cdc53 and Skp1. In addition, the last subunit can interact with several proteins characterized by the presence of a relatively conserved structural motif called F-box which is responsible for binding to Skp1 [4]. The resulting complex, called SCF for Skp1/Cdc53-cullin/F-box protein, acts as an E3 ubiquitin ligase that allowed the ubiquitination and subsequent proteolysis of Sic1 and Cln1,-2 by F-box proteins Cdc4 and Grr1 respectively in conjunction with E2 ubiquitin-conjugation enzyme Cdc34 and E1 ubiquitin-activation enzyme. Thus, F-box containing proteins, by virtue of their interaction with different target proteins and recruiting them for degradation, confer specificity to SCF complexes.

Recent data indicate that the SCF pathway is evolutionarily conserved since all the subunits of yeast SCF complex have orthologues in mammals. Human cullin 1 protein, a member of the Cdc53 family, selectively interacts with human Skp1 [5–8]. Only a limited number of mammalian proteins containing an F-box motif have been identified so far including cyclin F, Skp2, β-TrCP and NFB42 [9–12]. These proteins were shown to be associated with Skp1 thus forming specific SCF complexes [4,5,10–17]. The best studied SCF^{Skp2} complex includes the invariant core consisting of $p19^{Skp1}$ and cullin 1 associated with ubiquitin-conjugating enzyme Cdc34. The F-box component of the complex, $p45^{Skp2}$, binds to Skp1 via an F-box motif and due to the presence of C-terminal leucine-rich repeat (LRR) domain, it interacts with cyclin A-Cdk2 although some N-terminal sequences appeared also to be involved in this binding [5,13]. The abundance of Skp2 transcripts and protein and, consequently, Skp2-cyclin A-Cdk2 complex formation is greatly increased in many transformed cells and during S phase progression. In addition, inhibition of Skp2 expression prevented entry into S phase in both normal and transformed cells suggesting that this protein is an important component of the cell cycle control machinery [8,10].

We report here the identification of a structural homologue of Skp2, a protein containing an F-box motif and C-terminal LRRs. This protein, designated FBL2, interacts with Skp1 through its F-box domain. However, striking differences in expression and intracellular localization with reference to Skp2 argue for its distinct role.

2. Materials and methods

2.1. cDNA cloning and plasmid construction

The GenBank database of expressed sequence tags (ESTs) was searched for sequences related to the F-box region of Skp2 with the TBLASTN program [18]. ESTs matching to the F-box motif were retrieved and used to search overlapping ESTs. Obtained contigs of nucleotide sequences were then translated and analyzed for the presence of LRRs. One consensus sequence derived from overlapping rat ESTs encoding very likely a full-length protein was chosen for further analysis. The cDNA was obtained by polymerase chain reaction (PCR) amplification of reverse-transcribed rat hepatoma HTC cell RNA using the Advantage Polymerase mix (Clontech, Palo Alto, CA, USA) and the following primers: a sense primer (5′-CATGAG-

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Abbreviations: Cdk, cyclin-dependent kinase; EST, expressed sequence tag; GFP, green fluorescent protein; LRR, leucine-rich repeat; PCR, polymerase chain reaction

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GAGGGACGTGAACGGAGTG-3′) and an antisense primer (5′-CCTAAGCCGAGGGCAGTTCTGAC-3′). The partial rat Skp2 cDNA encoding the C-terminal part of protein, including F-box and leucine-rich repeats, and the full-length rat Skp1 coding sequence were amplified by PCR from reverse-transcribed rat hepatoma HTC and rat liver epithelial cell SDVI RNAs respectively using the following primers: 5′-CTTCCGGATGAGCTGCTCTTGGGAA-3′ and 5′-TAGACAACTGGGCTTTTGCAGAGTC-3′ for Skp2 and 5′-ATG-CCTACGATAAAGTTGCAGAGT-3′ and 5′-TCACTTGTCTTCA-CACCACTGGTT-3′ for Skp1. PCR products were cloned in pCRII vector (In Vitrogen, NV Leek, The Netherlands) and DNA sequencing was performed on both strands.

To generate an expression vector encoding the FLB2-GFP fusion protein, a *Bam*HI-*Xho*I fragment was released from pCRII-FBL2 and in-frame subcloned in the same sites of pEGFP-C2 (Clontech). The expression vectors encoding V5/6×His-tagged full-length FBL2 and N-terminal truncation mutants were constructed by PCR amplification of the corresponding region and cloning in the pcDNA3.1/V5-His vector (In Vitrogen). The identical Kozak consensus sequence CAC-CATG was incorporated at the 5′ ends of sense primers to ensure the equal translation of cloned fragments. All constructs were verified by DNA sequencing.

2.2. Determination of exon/intron organization and analysis of upstream regulatory sequences

The human genomic clone hCIT.131_K_1 (GenBank accession number AC005288) was used to elucidate the genomic organization of FBL2 by sequence comparison with FBL2 cDNA. The presence of splice donor and acceptor sites immediately flanking the alignment matches helped to predict the positions of exons. Based on the location of exon 1, the 2.7 kb upstream genomic sequence was searched for transcription factor binding sites by MatInspector [19].

2.3. Cell culture and transfections

Hepatocytes from adult male Sprague-Dawley rats were isolated by a two-step collagenase perfusion procedure. Hepatocytes were seeded in a mixture of 75% minimum essential medium and 25% medium 199, supplemented with 10% fetal calf serum (FCS) and, per ml: 100 IU penicillin, 100 mg streptomycin sulfate, 1 mg bovine serum albumin and 5 mg bovine insulin. After cell attachment (4 h later), the medium was renewed with the same medium deprived of FCS and supplemented with 1.4×10^{-6} M hydrocortisone hemisuccinate. Faza 967 and FAO, well-differentiated rat hepatoma cell lines derived from H4IIEC3, and poorly differentiated rat liver hepatoma cells HTC were grown in a mixture of 50% Ham's F-12 and 50% NCTC 135 and Dulbecco's modified Eagle's medium (DMEM), respectively, supplemented with 10% FCS. Rat liver epithelial cell lines SDVI and BRL 3A were maintained in Williams' E medium with 10% FCS. Human osteosarcoma cells U-2OS were grown in DMEM containing 10% FCS. U-2OS cells were transiently transfected using liposome-based transfection reagent DOTAP (Boehringer Mannheim).

2.4. RNA isolation and Northern blot analysis

Total RNA was extracted from adult rat tissue or cultured cells by the guanidinium thiocyanate procedure. Unless stated otherwise the cells were harvested at 60–70% confluence. 20 μg RNA was separated by electrophoresis through a 1.2% agarose gel in 2.2 M formaldehyde, 20 mM MOPS (pH 7.0), 10 mM sodium acetate, 1 mM EDTA and transferred onto a nylon membrane (Hybond N^+, Amersham) by capillary blotting. Hybridization was carried out in the presence of ^{32}P-labelled cDNA probe as previously described [20]. The equivalence of RNA loading was assessed by ethidium bromide staining.

2.5. Protein binding assay

[^{35}S]Methionine-labelled full-length FBL2, the deletion mutants and Skp1 were produced in vitro using the TNT T7 Coupled Reticulocyte Lysate System (Promega, Carbonnieres, France) according to the manufacturer's instructions. 5 μl FBL2 and its deletion mutant translation products were mixed with 7.5 μl Skp1 programmed lysates and diluted to 150 μl with binding buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM EDTA, 1 mM dithiothreitol and 5 μg/ml aprotinin, leupeptin, and soybean trypsin inhibitor). The mixture was incubated on ice for 30 min and was immunoprecipitated with 1 μg Penta-His antibody specific for 6×His tag (Qiagen, Courtaboeuf, France) or anti-V5 antibody (In Vitrogen) for 1 h at 4°C followed by incubation with protein G-agarose beads (Pierce, Rockford, IL, USA) for another 1 h. Beads were washed three times with binding buffer, then separated on a 15% SDS-polyacrylamide gel, fixed, dried and subjected to autoradiography.

3. Results

By using the sequence information derived from ESTs, the cDNA encoding a 276 amino acid F-box protein was isolated from rat hepatoma cells HTC (Fig. 1A). Analysis of the deduced amino acid sequence showed in addition to the F-box motif located at the N-terminal region, the presence of six perfect 26 amino acid LRRs in the C-terminal half of the protein (Fig. 1B). Thus, together with Skp2, this protein represents the second member of the subfamily of mammalian F-box proteins containing LRRs. Therefore it was named FBL2 for *F-B*ox/*L*eucine-rich repeat protein 2. The GenBank search revealed the presence of the gene for human FBL2 on the 137 496 bp clone hCIT.131_K_1 from human chromosome 17. The corresponding cDNA was obtained by PCR from reverse-transcribed human hepatoma Hep3B RNA and comparative analysis of amino acid sequence showed very high homology with the rat counterpart (more than 99% identity).

Comparison of the human FBL2 cDNA sequence to that of clone hCIT.131_K_1 allowed us to determine the exon/intron organization of the FBL2 gene (Table 1). Collectively, the data demonstrate that the FBL2 gene, covering almost the totality of the clone, is a large (at least 126.6 kb), highly interrupted gene composed of 10 small exons, most of them

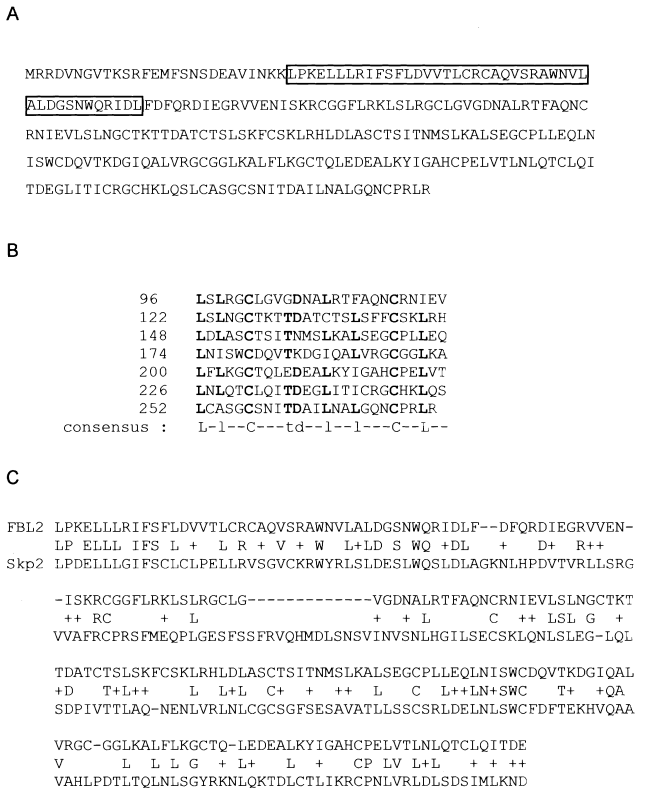

Fig. 1. Amino acid sequence of FBL2 and comparison with Skp2. A: Deduced amino acid sequence of rat FBL2. The F-box motif is indicated by a box. B: Alignment of leucine-rich repeat domains at the C-terminal region of FBL2. C: Sequence alignment of F-box motif and leucine-rich repeat domains of rat FBL2 and Skp2.

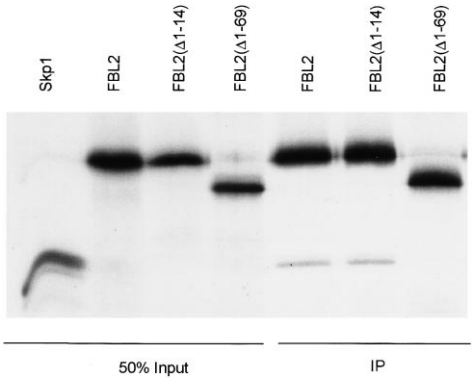

Fig. 2. FBL2 associates with Skp1 in vitro. 5 μl [^{35}S]methionine-labelled V5/6×His-tagged FBL2, deletion mutants FBL2(Δ1–14) and FBL2(Δ1–69) produced in rabbit reticulocyte lysates were mixed with 7.5 μl Skp1 programmed lysates in 150 μl binding buffer and immunoprecipitated with 1 μg anti-6×His antibody followed by incubation with protein G-agarose beads. Beads were washed three times with binding buffer and separated on a 15% SDS-polyacrylamide gel.

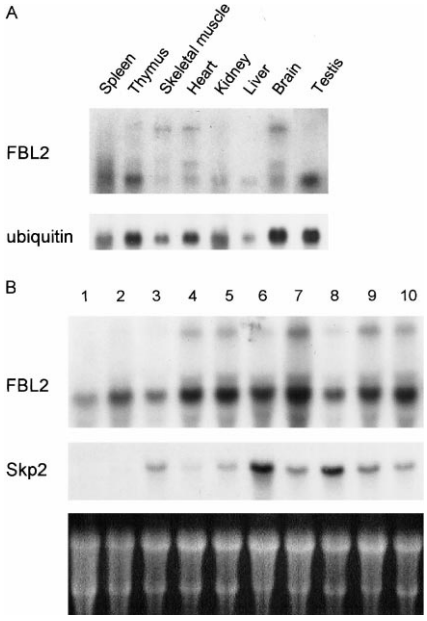

Fig. 3. Expression of FBL2 transcripts in adult rat tissues and in liver-derived cell lines. A: Total RNA was extracted from adult tissues indicated and subjected to Northern blot analysis using rat FBL2 cDNA probe. The equivalence of RNA loading was assessed by hybridization with a housekeeping gene ubiquitin cDNA probe. B: Total RNA was extracted from primary rat hepatocytes at 4 h (1) and 48 h of culture (2), from rat hepatoma cells HTC (3), Faza 967 (4) and FAO (5), from rat liver epithelial cells SDVI in the proliferative state (6) and maintained at confluence for 5 days (7), from the transformed variant of SDVI in the proliferative state (8) and at confluence (9) and from BRL 3A line (10) and analyzed by blot hybridization with rat FBL2 and Skp2 cDNA probes.

not exceeding 100 bp. The size of introns ranges from 1.3 to 58.1 kb. Analysis of splice donor/acceptor site sequences revealed that all intron/exon junctions followed the normal consensus sequence rules [21]. Most of the introns (five of nine) are type 0 introns, which interrupt the code between codons, and the remaining four introns are type 2 introns (codon interruption after the second nucleotide). Additionally, we found that the left extremity of the hCIT.131_K_1 clone contains the 3′ end of the thyroid hormone receptor-associated protein complex component TRAP220 gene, known also as thyroid hormone receptor interactor 2 (TRIP2), and peroxisome proliferator-activated receptor γ-binding protein (PBP)

Table 1
Exon-intron organization of FBL2

Exon No	Size (bp)	5′Splice donor	Intron No	Size (bp)	3′Splice acceptor
1	>176	TTT GAG gtg tga gct…	I	58.118	…ttt ttg cag ATG TTC
		F E^{14}			M F
2	62	TTA CG gta agt ctg…	II	39.540	…ctt ttt tag G ATA TTT
		L R^{35}			I F
3	55	TCC AGG gta agt att…	III	2.509	…tta aaa tag GCC TGG
		S R^{53}			G W
4	75	ATT GAG gta taa tta…	IV	1.926	…att tta cag GGC CGA
		I E^{78}			G R
5	95	TTA AG gta aaa aca…	V	1.795	…cct aca aag A ACC TTT
		L R^{110}			T F
6	69	GAC GC gta agt att…	VI	11.553	…att ttt cag T ACA TGT
		D A^{133}			T C
7	96	CTG AG gta aat ttc…	VII	2.621	…tat cct aag T GAG GGA
		L S^{165}			E G
8	127	ACG CAG gta ata ctc…	VIII	1.264	…tct ttc tag CTA GAA
		T Q^{207}			L E
9	75	TGC TTG gta aac atc…	IX	6.285	…gtt ttt tag CAA ATC
		C L^{232}			Q I
10	>160				

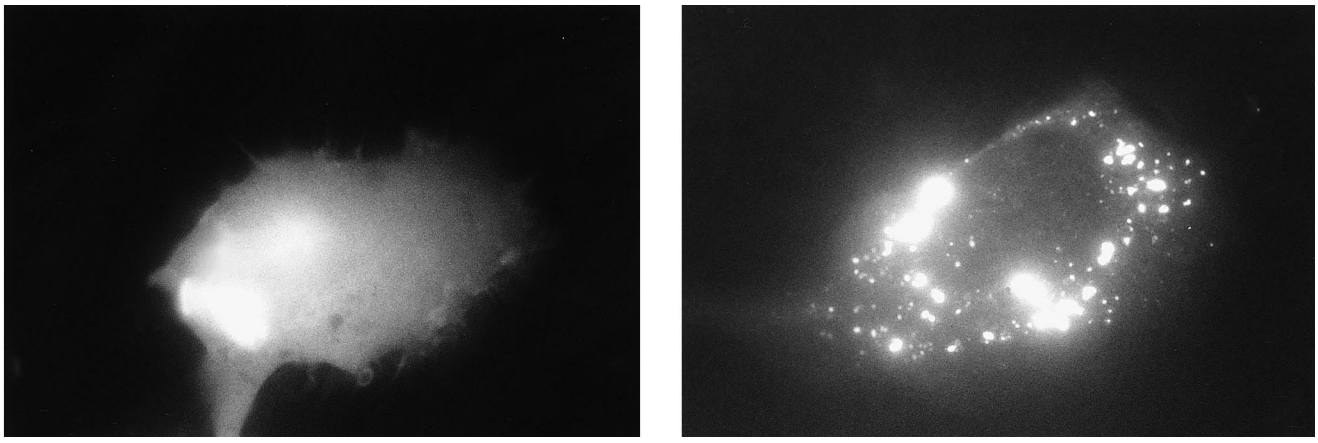

Fig. 4. Intracellular localization of FBL2. U-2OS cells were transiently transfected with expression plasmids encoding GFP (left panel) or FBL2-GFP fusion protein (right panel). 18 h after transfection the cells were fixed with PBS/4% paraformaldehyde and examined by fluorescence microscopy.

[22,23]. The 2.7 kb portion of the hCIT.131_K_1 clone separating the 3′ end of TRIP2 from the 5′ end of FBL2 was analyzed for the presence of promoter elements and transcription factor binding sites. By MatInspector search we detected a CCAAT box, two E-boxes and putative binding sites for Egr-1, Sp1, C/EBP and E2F transcription regulators.

Amino acid sequence similarity search of predicted protein FBL2 revealed a high homology with two *Caenorhabditis elegans* hypothetical F-box proteins C02F5.7 and C30A5.1 (GenBank accession numbers P34284 and AAB59171, respectively). As expected, significant homology was found with the C-terminal half of rat Skp2 encompassing the F-box and LRRs (Fig. 1C).

The presence of the F-box in FBL2 led us to investigate whether this protein could bind Skp1 as was shown previously for Skp2. [^{35}S]Methionine-labelled V5/6×His-tagged FBL2, deletion mutants FBL2(Δ1–14), FBL2(Δ1–69) and full-length Skp1 were mixed and immunoprecipitated with anti-6×His antibody. Both FBL2 and FBL2(Δ1–14) interact with Skp1, whereas FBL2(Δ1–69) lacking the F-box region failed to bind this protein (Fig. 2). Similar results were obtained with anti-V5 antibody (data not shown).

To gain insight into the possible role of FBL2, we performed a comparative analysis of its expression pattern and intracellular localization with that of Skp2. Total RNA was extracted from various adult rat tissues and analyzed by hybridization using full-length FBL2 cDNA as a probe. FBL2 mRNA appeared to be expressed ubiquitously, although its amount varied considerably among the tissues tested. At least three mRNA transcripts were detected in analyzed tissues with low sized species enriched in spleen, thymus and testis and high molecular weight transcripts were predominantly present in skeletal muscle, heart and brain. The liver expressed the lowest amounts of transcripts (Fig. 3A). Furthermore, expression of FBL2 was analyzed in a set of liver-derived cells, including primary hepatocytes and established cell lines with different degrees of tumorigenicity and differentiation. The gene was expressed in freshly isolated hepatocytes and increased with time in culture in the absence of mitogen stimulation. FBL2 mRNAs were detected in all liver-derived cell lines analyzed; however, well-differentiated hepatoma cells FAO and Faza 967 contained higher amounts of transcripts in comparison to dedifferentiated HTC cells. In addition, expression of FBL2 was related to proliferation status of the cells. In both normal and transformed rat liver epithelial cells the amount of mRNA increased in growth-arrested confluent cells. In contrast, Northern blot analysis of Skp2 expression revealed a completely opposite pattern: Skp2 transcripts were undetectable in non-proliferating primary hepatocytes, abundant in proliferating hepatoma and rat liver epithelial cells and down-regulated at confluence (Fig. 3B).

To examine the intracellular localization of FBL2, it was expressed as a GFP fusion protein in U2-OS cells. The vector pEGFP-C2 encoding enhanced GFP was used as a control. Analysis of transfected cells by fluorescence microscopy revealed that in contrast to EGFP, which showed a homogeneous intracellular pattern of fluorescence, the fluorescent signals in cells transfected with the FBL2-GFP construct were detected in punctuate foci in the cytosol concentrated mainly around the nuclei (Fig. 4).

4. Discussion

Originally identified as a protein associated with complexes of the cell cycle regulators cyclin A and Cdk2, Skp2 was found to be a component of SCF-type E3 ligases which function in the ubiquitin-dependent destruction of target proteins. Due to the presence of the F-box domain Skp2 recruits substrates to the invariable core of E3 ligases which include Skp1 and Cul-1. To identify new Skp2-like proteins we performed a computational similarity search against EST databases for cDNA encoding proteins containing the F-box motif and leucine-rich repeats. Using this strategy, we discovered and cloned an additional member of F-box family, a 276 amino acid protein which contains six LRRs, and determined the exon/intron organization of the human FBL2 gene located on chromosome 17.

To investigate whether FBL2 could form an SCF complex, we tested its ability to interact with Skp1. We provided evidence that the full-length FBL2 protein and an N-terminal 14 amino acid deletion mutant interact with Skp1, while the F-box deletion mutant was defective for Skp1 binding. Thus our data again support the conclusion that the F-box motif is indispensable for association with Skp1 and that FBL2 also uses this motif to form an SCF^{FBL2} complex.

One might expect, based on the presence of similar func-

tional domains, F-box and LRRs, in both Skp2 and FBL2, that these two proteins could have at least related functions. However, the FBL2 expression pattern was found to be different from and even opposite to that of Skp2. Indeed, it was demonstrated that expression of Skp2 is increased in transformed cells and during S phase of the cell cycle [5,8,10]. Skp2 was also induced in regenerating rat liver in vivo and growth factor-stimulated normal hepatocytes in vitro [24]. Consistent with previous reports we found that Skp2 was strongly expressed in proliferating liver-derived cell lines and down-regulated in quiescent cells. In contrast, FBL2 transcripts were preferably expressed in confluent growth-arrested cells. In addition, the nuclear localization of Skp2 [5] was clearly different from that of FBL2, which was detected in the cytosol around nuclei.

Taken together our data suggest that despite the large structural homology with Skp2 and similar capacity to bind Skp1, FBL2 likely plays a distinct role as argued by the pattern of its expression in liver-derived cells and intracellular localization, the latter probably reflecting an association with different target proteins. Indeed, since the binding partners of Skp2, cyclin A and cdk2, are predominantly nuclear during S phase this could explain the nuclear co-localization of the associated SCF^{Skp2} complex. In contrast, it seems that yet unidentified protein(s) binding to SCF^{FBL2} could target FBL2 to the perinuclear compartment. In line with this possibility we failed to demonstrate binding of FBL2 to cyclin A-Cdk2 from regenerating rat liver using the same assay conditions that have previously allowed us to detect the association of Skp2 with this complex (unpublished data).

To date, besides FBL2, five F-box subunits of SCF complex have been identified in mammals: cyclin F, Skp2, highly homologous β-TrCP1 and β-TrCP2 (KIAA 0696) and NFB42. In addition to the F-box motif, these proteins contain different C-terminal domains, known to be involved in protein-protein interactions. Namely, cyclin F includes a cyclin box [9], β-TrCP1 and β-TrCP2 contain WD40 repeats [11,14,25], the C-terminal half of NFB42 shares homology with *C. elegans* hypothetical protein C14B1.3 [12] and, finally, Skp2 and FBL2 contain LRRs. Due to the presence of an F-box all these proteins are able to associate with Skp1, thus forming the core of the E3 ubiquitin ligase protein complexes which could recruit specific substrates for degradation. Up to now, this role was clearly established for β-TrCP only, which is involved in ubiquitin-dependent proteolysis of β-catenin and IκBα [14–17,25,26]. The presence of an F-box in FBL2, which is indispensable for binding of Skp1, strongly suggests that this protein could interact with and recruit protein(s) for ubiquitination. Studies are in progress to identify proteins that associate with FBL2 and to define its role in the ubiquitin-proteasome pathway.

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