Occurrence of a Putative SCF Ubiquitin Ligase Complex in Drosophila

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Many proteins are targeted to proteasome degradation by a family of E3 ubiquitin ligases, termed SCF complexes, that link substrate proteins to an E2 ubiquitin-conjugating enzyme. SCFs are composed of three core proteins—Skp1, Cdc53/Cullin, Rbx1/Hrt1—and a substrate specific F-box protein. We have identified in Drosophila melanogaster the closest homologues to the human components of the SCFβTrCP complex and the E2 ubiquitin-conjugating enzyme UbcH5. We show that putative Drosophila SCF core subunits dSkpA and dRbx1 both interact directly with dCu11 and the F-box protein Slmb. We also describe the direct interaction of the UbcH5 related protein UbcD1 with dCul1 and Slmb. In addition, a functional complementation test performed on a Saccharomyces cerevisiae Hrt1p-deficient mutant showed that Drosophila Rbx1 is able to restore the yeast cells viability. Our results suggest that dRbx1, dSkpA, dCullin1, and Slmb proteins are components of a Drosophila SCF complex that functions in combination with the ubiquitin conjugating enzyme UbcD1.

Key Words: SCF; ubiquitin; Drosophila; proteolysis; E3 ligase.

The ubiquitin/proteasome pathway plays a key role in many basic cellular processes (reviewed in 1). In addition to degradation of defective or misfolded proteins, a critical regulatory role for this system has been defined in studies of the cell cycle. Some major signal transduction pathways, which are of great importance in development and cell differentiation, are known to be controlled in the same way. How do eukaryotic cells decide which proteins are due to enter the ubiquitin/proteasome pathway and when such proteins should do so? Typically, the conjugation reaction occurs in three successive steps. First, Ubiquitin Activating Enzyme (E1) hydrolyzes ATP to form an AMP-ubiquitin adenylate. After release of AMP, activated ubiquitin is transferred to the active site cysteine of E1 to form an E1-thiolester bond. Second, activated ubiquitin is transferred from E1 to an Ubiquitin Conjugating Enzyme (E2) in a reaction in which the thiolester-bond is transferred. Finally, a Protein Ubiquitin Ligase (E3) transfers ubiquitin from E2 to a lysine group on the target protein. A multi-ubiquitin chain is generated, targeting the protein for degradation by the 26S proteasome.

Results from several laboratories have demonstrated that ubiquitin conjugation is the step that defines target specificity for degradation, and that E3 protein-ubiquitin ligases are the enzymes responsible for such specificity. Although some E3 enzymes are single polypeptides, multienzyme complexes appear to be most common. Among these complexes, SCF E3 ubiquitin ligases have been well characterized in mammalian and yeast systems (2). They are composed of three constant polypeptides—Skp1, Cdc53/Cullin1 (Cu1) and Rbx1/Hrt1—and one variable component (F-box protein), which is specific to a particular target or small group of target proteins. Evidence provided by several laboratories indicate that F-box proteins behave as adapters between substrates and the rest of the complex (3). The so-called "Combinatorial F-box Hypothesis" proposes that many different substrates can be recognized by various SCF complexes that differ in their F-box adapter protein (3–5). A mammalian SCF E3 ubiquitin ligase complex that contains an F-box protein named βTrCP controls NFκB and Wnt/Wingless signaling pathways (6). Such control is exerted through ubiquitination of IκB and β-catenin, respectively thus, targeting them for 26S proteasome degradation. Although the existence of SCF complexes has not been demonstrated in Drosophila, database sequences analysis indicates that many F-box proteins...
occur in the fruit fly. Slmb (Slmb), the Drosophila homologue of βTrCP, is a negative regulator of Wingless and Hedgehog signaling pathways during limb development (7, 8) and also seems to control NFκB pathway (9). Slmb loss of function mutations result in a cell-autonomous accumulation of high levels of Cubitus interruptus (Ci), a transcription factor that activates the expression of Hedgehog responsive genes and Armadillo (Arm), the Drosophila β-catenin homologue (8). These results suggest that Slmb plays an analogous role to mammalian βTrCP, targeting Arm, Ci, and Cactus (the IκB Drosophila homologue) for ubiquitination and proteolysis by the 26S proteasome. In addition, Slmb was also shown to be required for proper centrosome duplication in Drosophila causing the appearance of additional centrosomes and mitotic defects in mutant larval neuroblasts (10).

Recently, a cul1/cdc53 Drosophila homologue gene has been described (11) and several sequences homologous to vertebrate and yeast skp1 and rbx1/hrt1 are present in the Berkeley Drosophila Genome Project database. Nevertheless, no biochemical evidences for the occurrence of SCF complexes in Drosophila were presented so far. In this report, we provide evidence for the existence of a Slmb-containing SCF complex in the fruit fly by utilizing the yeast two-hybrid system as well as in vitro interactions. In addition, we tested the ability of skpA, cul1, and rbx1 Drosophila genes (dskpA, dcul1, and drbx1) to complement the corresponding null mutations in Saccharomyces cerevisiae. We found that drbx1, but not the other two components of the Drosophila SCF complex, functionally complements dhr1, thus indicating that Rbx1 is largely conserved in evolution.

MATERIALS AND METHODS

DNA database searches. Drosophila sequences with the highest identity to the human proteins Skp1, Rbx1, and Cul3 (GenBank Accession Nos: U33760, AF140598, and AF062537, respectively) were identified at the Berkeley Drosophila Genome Project (BDGP) database using BLAST and designated as dSkpA, dRbx1, and dCul3. The identification of dCul1 was recently reported (11). To obtain the corresponding cDNAs, the BDGP Drosophila EST database was searched and several Drosophila EST clones were found to encode proteins matching the 5′ end of the identified sequences. cDNA clones corresponding to the Drosophila F-box protein Slmb (AF032878), dCul1 (Q24311), and the Drosophila E2 ubiquitin-conjugating enzymes UbcD1 (12) (X62575), UbcD6 (13) (M63792), and UbcD10 (14) (A238007) were obtained from the same source. One representative EST clone was selected between those matching the cDNA sequences corresponding to dSkpA (Clone ID: LP02056), dRbx1 (Clone ID: GH12110), dCul1 (Clone ID: LD29973), dCul3 (Clone ID: GH27418), Slmb (Clone ID: LD31459), UbcD2 (Clone ID: LD32571), UbcD6 (Clone ID: LD22830), and UbcD10 (Clone ID: LD30207) and used for experiments. Clones were sequenced by automated DNA sequencing to confirm that all ESTs contained the full-length coding region. The sequences of dSkpA, dRbx1, and dCul3 correspond to the Flybase annotations: gene CG16983, (transcript CT40292), gene CG16982 (transcript CT32695), and gene CG11861 (transcript CT35206), respectively.

Plasmid construction. cDNAs were amplified by PCR using oligonucleotides that introduced restriction enzyme sites for in-frame cloning of the PCR products into pTrChisC (Invitrogen) or pGEX-3X plasmids (Pharmacia).

In vitro transcription and translation. [35S] methionine-labeled proteins were produced from ESTs cloned in the pOT2 vector by coupled in vitro transcription and translation (IVTT) reactions in rabbit reticulocyte lysate (TNT, Promega).

Expression of recombinant proteins in E. coli. Cultures of E. coli DH5α bearing GST- or His-fusion protein encoding plasmids were grown to an A600 of 0.5. Induction was performed by addition of IPTG and was added to a final concentration of 50–100 μM. Cultures were allowed to grow for 2.5 h at 28°C, and cells were harvested by centrifugation at 4°C.

Purification of GST-fusion proteins. Bacterial pellets were lysed on ice by sonication in resuspension buffer (PBS, 1% Triton X-100, 0.5 mM EDTA) containing a protease inhibitor cocktail (0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml Leupeptin, 1 μg/ml Pepstatin, and 1 μg/ml Aprotinin). Lysates were centrifuged, supernatants mixed with glutathione-agarose beads (Sigma) (200 μl beads per 50 ml of initial bacterial culture) were allowed to bind for 1 h at 4°C. Glutathione-agarose beads were pelleted by low speed centrifugation and washed five times in resuspension buffer. For binding assays (below), the concentration of GST fusion proteins in the slurry of glutathione-agarose beads was estimated by SDS-PAGE and Coomassie blue staining.

Glutathione-agarose pull-down assay. GST-fusion proteins bound to glutathione-agarose (–20 μl beads) were mixed with 2 μl of [35S] methionine-labeled protein generated by IVTT, and allowed to interact at 4°C for 3 h in 100 μl of EBC buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% w/v gelatin, 0.5 mM EDTA). Glutathione-agarose beads were pelleted by low speed centrifugation and washed five times in EBC buffer at 4°C. Samples were resuspended in SDS-sample buffer and associated proteins were detected by SDS-PAGE followed by fluorography.

Purification of His-fusion proteins. Bacterial pellets were lysed on ice by sonication in binding buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 5 mM imidazole) with a protease inhibitors cocktail. Lysates were centrifuged and the supernatants were mixed with imidodiacetic acid-sepharose beads (Sigma) previously charged with Ni2+. The beads were washed with 10 volumes of binding buffer followed by 6 volumes of washing buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 60 mM imidazole). Bound His-fusion proteins were recovered with 6 volumes of elution buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 1 M imidazole).

In vitro interaction assays using His-tagged proteins. His-tagged purified proteins (100 μl) were mixed with 4 μl of rabbit reticulocyte lysate containing [35S] methionine-labeled protein and 400 μl of EBC buffer and allowed to interact for 3 h at 4°C. Samples were incubated for 2 h at 4°C with 200 ng of anti-His antibody (H-15) (Santa Cruz Biotechnology) and immunoprecipitation was performed by addition of 20 μl of protein A-Sepharose (Sigma) followed by low speed centrifugation. After washing the beads five times in EBC buffer, samples were dissolved in sample buffer and associated proteins were detected by SDS-PAGE followed by autoradiography.

 Yeast two-hybrid interactions. pAS2-1 plasmid (Clontech) was used to generate fusion proteins containing the GAL4 DNA-binding domain (DNA-BD) at their N-terminal end and pACT2 vector (Clontech) to generate proteins containing the GAL4 activation domain (AD) with a hemagglutinin (HA) peptide at their N-terminal end. Expression in yeast was confirmed by Western blot using anti-GAL4DBD (IKE) or anti HA-probe (Y-11) antibodies (Santa Cruz Biotechnology) (data not shown). The P J X yeast strain that contains three reporter elements (HIS3, ADE2, and LacZ) was transformed with various combinations of recombinant pACT2 and pAS2-1 plasmids and plated on SD medium lacking leucine and triptophane to select
constructed on strain YPH274. SKP1 fragments to generate heterozygous diploids with null alleles of YPH274 (ATCC, Rockville, MD) was also transformed with PCR (SKP1/skp1::HIS3), and SWY200 (HRT1/). Diploid strain hrt1DD D trophic using the PCR products to generate strains SWY100 and SWY200. Diploid strain CEN.PK2 was transformed to histidine prototrophy using the pFA6a-HIS3MX6 plasmid used for PCR-based gene disruptions was provided by Dr. Peter Philippsen (University of Basel, Switzerland). The sequence with highest homology to the human protein designated as dSkpA (GadFly annotation CG16983) was found 76% identical to hSkp1 whereas the other Drosophila Skp1 related sequences showed lower degrees of identity (ranging between 17 and 67%). dSkpA is also the closest Drosophila homologue of the yeast Skp1 protein and the putative C. elegans Skp1 (Fig. 1). A similar BLAST search was performed using the human Rbx1 protein sequence against the BDGP database and three Drosophila sequences were found with significant homology to the human protein. The sequence corresponding to the GadFly annotation CG16982, designated as drbx1, presents the highest degree of conservation with hRbx1 (91% identity) (18). The other two Drosophila Rbx-related sequences are 70% (CG16988) and 30% (CG18042) identical to hRbx1. Nevertheless, CG18042 shows the highest identity (70%) with the APC11 anaphase-promoting complex subunit (19). In mammalian and yeast cells, the third core component of SCF complexes is a member of the cullin family, Cul1. In Drosophila, seven members of this family can be found. The Drosophila orthologue of human Cul1 has been recently described as a cell-cycle regulator named dc1 (11), which is 62% identical to the human protein. Another Drosophila cullin family member (GadFly annotation CG1861) is highly conserved with human Cul3 (67% identity), a protein implicated in cyclin E ubiquitination (20). Once the components of a putative Drosophila SCF complex were identified, we employed the yeast two-hybrid system to investigate interactions between Genome Project (BDGP) database with the human Skp1 and Rbx1 protein sequences. BLAST search using hSkp1 yielded 7 Drosophila Skp1-related proteins. In Drosophila, most organisms have several other Skp1 family members, but in all cases their function remain largely unknown (17). The sequence with highest homology to the human protein designated as dSkpA (GadFly annotation CG16983) was found 76% identical to hSkp1 whereas the other Drosophila Skp1 related sequences showed lower degrees of identity (ranging between 17 and 67%). dSkpA is also the closest Drosophila homologue of the yeast Skp1 protein and the putative C. elegans Skp1 (Fig. 1). 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Table 1

Specific Interactions in the Two-Hybrid System

<table>
<thead>
<tr>
<th>Gal4AD-hybrid (pACT2)</th>
<th>Gal4BD-hybrid (pAS2-1)</th>
<th>His/Ade</th>
<th>β-Gal</th>
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<td>dSkpA</td>
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<td>+</td>
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<tr>
<td>dCul1</td>
<td>dSkpA</td>
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<td>dSkpA</td>
<td>dCul3</td>
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<td>UbcD10</td>
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<td>UbcD10</td>
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<td>dRbx1</td>
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<tr>
<td>Slmb</td>
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<td>Slmb</td>
<td>dSkpA</td>
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Note. Plasmids expressing the indicated fusion proteins were cotransformed into yeast cells. Three independent colonies for each transformation were streaked on SD-Leu-Trp-Ade-His selective medium containing X-Gal. Interactions between fusion proteins were assessed by growth on the selective medium, which indicates the activation of the ADE2 and HIS3 reporter genes, and by their ability to induce activation of the LacZ reporter gene recorded by blue color staining.

In order to increase sensitivity, the interactions tested as negative in the plate assay were retested in liquid assays for β-galactosidase activity. With this assay, two new interactions were recorded as positive: UbcD1-dCul1 and UbcD1-dCul3. Among the three Drosophila E2s conjugating enzymes tested, only UbcD1 interacts with the cullins dCul1 and dCul3 while no interactions were detected between this cullins and UbcD6 or UbcD10, indicating that E2s are probably not interchangeable. Multiple E2s have been reported in diverse organisms, and these likely possess specificity for different classes of target proteins (24). Drosophila UbcD1 appears to influence the degradation of a wide array of proteins (12, 25–27) and can functionally complement the yeast UBC4 mutant phenotype (12). Consistently, UbcD1 gene shows the highest sequence similarity to yeast Ubc4/Ubc5 (79% identity) and human UbcH5 (94% identity), and E2 enzyme implicated in SCF-μ-TRCP-dependent degradation of IκB (25). Several other E2 enzymes exist in Drosophila showing lower degrees of identity with UbcH5 (32 to 66%).

dRbx1-Gal4 DNA-binding domain fusion protein (bait) rendered strong transcriptional activation even in the absence of a prey. Similarly, dRbx1 placed as a prey brought about activation in the absence of a bait. Therefore, interactions with dRbx1 could not be assessed by the two hybrid system and were evaluated in vitro. A glutathione-S-transferase (GST)-tagged dRbx1 protein was prepared by fusing GST to its N-terminal and expressed in bacteria. Interactions of GST–dRbx1 with different proteins generated by in vitro transcription and translation (IVTT) were assessed by the GST pull down assay. As shown in Fig. 2A, dRbx1 interacts specifically with dCul1 and the F-box protein Slmb but fails to interact with dSkpA. This is consistent with results reported in yeast where Hrt1 binds Cdc53 (29) and the F-box proteins Grr1 and Cdc4 (18). In accordance with our result in Drosophila, no direct interaction between Skp1 and Rbx1 was reported in any organism. dRbx1 did not show self-interaction in the GST-pull down assay probably reflecting the fact that it is present in the complex as a monomer (Fig. 2A). Similar in vitro experiments were performed in order to confirm the interactions assessed through the yeast two-hybrid system. For this purpose His-tagged dSkpA and UbcD1 proteins were generated by fusing six histidine residues to the N-terminus of dSkpA and UbcD1. Both proteins were expressed in bacteria and purified using an iminodiacetic acid-sepharose column charged with Ni²⁺. Figure 2B shows that radiolabeled Slmb and dCul1, but not dRbx1, generated by IVTT specifically interact with the His-dSkpA and His-UbcD1 chimeras. These results confirm the ones obtained in the two-hybrid system and add the observation of a direct interaction between UbcD1 and Slmb. We observed that Cull1 and Slmb migrate as a doublet (Fig. 2). Cull1 double band could be due to conjugation to endogenous Nedd8/Rub1 present in the reticulocyte lysate. Nedd8/Rub1 was shown to be covalently attached to yeast Cdc53 and to all human cullin-family proteins resulting in appearance of a higher molecular mass polypeptide (30, 31). Slmb doublet could be due to some unknown different posttranslational modification.
We next examined whether Drosophila dCul1, dSkp1, and dRbx1 genes are able to functionally complement the lethal phenotype of yeast strains lacking cdc53, skp1, and hrt1, respectively. Tetrad analysis of heterozygous disruptant strains CSLZ019(HE), SWY100, and SWY200 was performed. For each tetrad dissected, only two viable colonies were produced, both of which were histidine auxotrophs. Consistent with the essential nature of these genes, only his² spores gave rise to normal size colonies, whereas inviable spores formed microcolonies of 4–8 multibudded and elongated cells.

Drosophila Rbx1 rescued the lethal phenotype of the Δhrt1 yeast strain. Tetrad analysis of strain SWY200 transformed with pYes2-dRbx1 yielded 2, 3, and 4 viable spores on plates with galactose. Consistent with this, his⁻ viable spores were only obtained on galactose, but not on glucose plates, and were always ura⁻, indicating that the lethal phenotype of Δhrt1 was suppressed by expression of Drosophila Rbx1 (Fig. 3A).
Haploid strains Δhrt1 rescued by pYes2-dRbx1 on plates with galactose, grew on liquid medium containing galactose with division times comparable to those of the wild-type strain. The ability of the complemented strain to grow on liquid medium was strictly dependent on the expression of the Drosophila gene. When cultures of this strain that were growing exponentially in liquid medium with galactose were shifted to the same medium containing glucose, a rapid arrest in growth was observed due to repression of the heterologous gene (data not shown). In addition, repression of Drosophila dRbx1 by growth in glucose caused the yeast cells to exhibit the characteristic phenotype of hrt1-deficient yeast strains (32) i.e., accumulation of elongated cells with multiple abnormally shaped buds (Fig. 3B). The ability of dRbx1 to complement Δhrt1 yeast phenotypes is similar to the reported complementation by the murine or human Rbx1 homologues (18, 33, 34) and demonstrates the functional conservation of dRbx1 in Drosophila.

dCul1 and dSkpA failed to complement the lethal phenotype of the corresponding null mutations in S. cerevisiae although the complementation tests were performed using constructs bearing the Drosophila cDNAs under control of two different promoters in two different yeast strains (see Materials and Methods). The lack of functional complementation of the cdc53 strain may be due to the low level of identity between the Drosophila and yeast proteins (28%). In accordance with our data, hCul1 does not complement a cdc53 null mutant yeast strain although it complements a thermo-sensitive cdc53 mutation (21). On the other hand, even though the overall conservation between dSkpA and yeast Skp1 is higher (55% identity), the recent crystal structure of a human Skp1 containing complex suggests that divergences in its variable C-terminal helix, involved in the recognition of specific subsets of F-box proteins, could be responsible for the lack of complementation (17). In this work, other members of the Drosophila Skp1 family were not assayed for the ability to interact with components of the SCF complex or to complement the Skp1 yeast null mutant. Thus, we can not rule out the possibility that one of such proteins is the actual Skp1 functional homologue.

Our results, summarized in Fig. 4, provide evidences that dRbx1, dSkpA, and dCul1 are components of a Drosophila SCF complex containing the F-box protein Slmb that functions in combination with the ubiquitin conjugating enzyme UbcD1. Further characterization of this SCF complex will require the demonstration of its ubiquitin ligase activity.

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