Pseudosubstrate regulation of the SCF ^{β -TrCP} ubiquitin ligase by hnRNP-U

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β -TrCP/E3RS (E3RS) is the F-box protein that functions as the receptor subunit of the SCF ^{β -TrCP} ubiquitin ligase (E3). Surprisingly, although its two recognized substrates, I κ B α and β -catenin, are present in the cytoplasm, we have found that E3RS is located predominantly in the nucleus. Here we report the isolation of the major E3RS-associated protein, hnRNP-U, an abundant nuclear phosphoprotein. This protein occupies E3RS in a specific and stoichiometric manner, stabilizes the E3 component, and is likely responsible for its nuclear localization. hnRNP-U binding was abolished by competition with a pI κ B α peptide, or by a specific point mutation in the E3RS WD region, indicating an E3–substrate-type interaction. However, unlike pI κ B α , which is targeted by SCF ^{β -TrCP} for degradation, the E3-bound hnRNP-U is stable and is, therefore, a pseudosubstrate. Consequently, hnRNP-U engages a highly neddylated active SCF ^{β -TrCP}, which dissociates in the presence of a high-affinity substrate, resulting in ubiquitination of the latter. Our study points to a novel regulatory mechanism, which secures the localization, stability, substrate binding threshold, and efficacy of a specific protein-ubiquitin ligase.

[Key Words: β -TrCP/E3RS; hnRNP-U; I κ B α ; nuclear transport; ubiquitin ligase]

Received October 15, 2001; revised version accepted December 15, 2001.

Ubiquitin-mediated protein degradation is a highly selective process that involves the concerted action of several enzymes to assign a protein for degradation (Varshavsky 1997; Hershko and Ciechanover 1998). Substrate recognition is carried out by the versatile set of E3 proteins (Laney and Hochstrasser 1999). Among the latter, one of the better-defined E3s is SCF ^{β -TrCP}, the E3 complex that targets pI κ B α and β -catenin for degradation (for reviews, see Maniatis 1999; Po-lakis 1999; Karin and Ben-Neriah 2000). Our pre-vious work showed that pI κ B α is recognized by the β -TrCP/E3RS subunit of the SCF ^{β -TrCP} via a short, linear, doubly phosphorylated peptide (Yaron et al. 1997, 1998).

SCF-type E3s are assemblies of several common (Skp1, Cul1, and Roc1/Rbx1/Hrt1) and single-variable (F-box protein) protein components, which were discovered and have mainly been characterized in yeast (Patton et al. 1998; Deshaies 1999; Jackson et al. 2000). Genes encoding certain SCF subunits are essential to cell cycle progression, and mutations in the different subunits result in a similar phenotype of cell cycle arrest, supporting the

view that they are acting in concert. Many substrates of these E3 ligases share a common feature: phosphorylation as a prerequisite for recognition by the ligase. Having no apparent catalytic function of their own, SCF ligases rely on E2s for facilitating the covalent attachment of ubiquitin to the substrate. With the exception of the F-box proteins, the function of other SCF subunits has been only partially resolved (Deshaies 1999). At least one subunit, Skp1, is thought to serve as an adapter that links the F-box protein to the rest of the complex. The other subunits, Cul1 and the newly discovered subunit ROC1/Rbx1/Hrt1, may act to recruit an E2 to the substrate through a motif called the R-box (a RING finger, small metal-binding domain), and are involved in the polymerization of the ubiquitin chain (Ohta et al. 1999; Seol et al. 1999; Skowyra et al. 1999; Tan et al. 1999; Jackson et al. 2000). Polyubiquitination is a signal for engaging the 26S proteasome and targeting substrates for rapid degradation. Although there has been rapid progress in the biochemical characterization of the SCF-type ligases, many aspects of their function remain mostly obscure, particularly the developmental and cellular regulation of SCF complexes in multicellular organisms. This report describes a novel SCF regulatory process in mammalian cells that acts via the engagement of a pseudosubstrate.

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.218702>.

Results

E3RS resides predominantly in the nucleus

Present models imply that the major function of I κ B α degradation is the exposure of the NF- κ B nuclear localization signal (NLS), resulting in binding to importins and karyopherins and translocation of NF- κ B from the cytoplasm into the nucleus (Karin and Ben-Neriah 2000). Accordingly, one would suppose that the ubiquitin ligase operates in the cytoplasm. Surprisingly, however, Western blot analysis of nuclear and cytoplasmic fractions from HeLa and 293 cells showed that endogenous E3RS was detected in the nucleus (Fig. 1A): anti-E3RS antibodies reacted exclusively with a 62-kD band in the nuclear fraction. Immunostaining of a Flag-tagged exogenously expressed E3RS gave a predominant nuclear signal (Fig. 1B). According to ProfileScan (SWISS-PROT) analysis, the amino acid sequence of E3RS has no apparent canonical nuclear localization motif (NLS). Therefore, its presence in the nucleus could be secondary to an associated protein, such as a component of the SCF complex. However, only a minor fraction of the transfected E3RS was associated with other SCF components (data not shown), making it unlikely that E3RS is carried into the nucleus by the SCF complex. Of note is the finding that β -TrCP2/HOS, the closest homolog of E3RS, which, similarly to E3RS, is assembled into an SCF complex (Fuchs et al. 1999; Suzuki et al. 2000), was found mainly in the cytoplasm (Fig. 1C).

Identification of hnRNP-U as the major E3RS-associated protein

To identify the E3RS-transporting protein, Flag-tagged E3RS was immunopurified from overexpressing cells and analyzed by SDS-PAGE (Fig. 2A) and mass spectrometry. A protein with the apparent molecular mass of 120 kD (p120) was specifically associated with E3RS at a near stoichiometric ratio (Coomassie staining ratio of 1.5:1 for p120/E3RS; Fig. 2A, lane 1). Neither Skp2 (Fig. 2A, lane 6), another SCF-associated human F-box protein (Krek 1998), nor β -TrCP2/HOS (Fig. 2A, lane 3), coimmunopurified with a similar 120-kD protein. Mass spectrometry sequencing of the major E3RS-associated protein identified it as hnRNP-U (Pinol-Roma et al. 1988; Kiledjian and Dreyfuss 1992), or scaffold attachment factor A (SAF-A; Romig et al. 1992), an abundant nuclear protein. The specificity of hnRNP-U association was confirmed by Western blot with monoclonal anti-hnRNP-U antibodies (Fig. 2B): the protein signal was detected in association with E3RS, but not with β -TrCP2/HOS or Skp2. Furthermore, human E3RS interacted with Flag-hnRNP-U of human and mouse origin (Fig. 2C), indicating a conserved association. As no other protein coprecipitated with E3RS at an appreciably high level (Fig. 2A), the exclusive association of transfected E3RS and endogenous hnRNP-U at stoichiometric proportions must represent a direct interaction.

E3RS contains two protein-protein interaction modules, the F-box and the WD40-repeat domain (Margottin

Figure 1. E3RS is localized predominantly in the nucleus. (A) Western blot analysis of endogenous E3RS was performed with cytoplasmic (C) and nuclear (N) fractions from HeLa and 293 cells, using goat anti- β -TrCP/E3RS (C18, Santa Cruz). c-Myc and I κ B α were used as references for the two cellular fractions. (B,C) CHO cells were transfected with (B) Flag-E3RS or (C) Flag-HOS/ β -TrCP2 and were stained with primary anti-Flag antibodies and rhodamine goat anti-mouse secondary antibodies.

et al. 1998; Yaron et al. 1998). To determine which module was responsible for binding hnRNP-U, fragments composed of the F-box or the WD40 repeat (an F-box-deleted E3RS) were expressed separately, immunopurified, and analyzed for hnRNP-U binding. hnRNP-U copurified with the WD40 repeat (Fig. 2A, lane 2, and B), but not with the F-box fragment (Fig. 2A, lane 4, and B). Should hnRNP-U be involved in regulating the subcellular localization of E3RS, it would only affect the localization of the WD module. Indeed, immunostaining of cells expressing the separate E3RS modules indicated the distinct position of the two: whereas the WD module was found in the nucleus, the F-box fragment was cytoplasmic (Fig. 2D).

To determine which part of hnRNP-U interacts with E3RS, several hnRNP-U fragments were prepared and examined for E3RS binding in transfected cells (Fig. 3A). A 198-amino-acid N-terminal fragment of hnRNP-U, composed of long acidic (33% Glu and Asp) and short glutamine-rich peptide segments (Pinol-Roma et al. 1988; Kiledjian and Dreyfuss 1992), was found to interact with E3RS similarly to the intact protein, or to a 400-amino-acid N-terminal fragment (Fig. 3B). The reciprocal 600-amino-acid C-terminal fragment, containing the RNA-binding region (Pinol-Roma et al. 1988; Kiledjian and Dreyfuss 1992), bound only trace E3RS levels.

The subcellular localization of E3RS is likely determined by hnRNP-U

To find out if the observed interaction between E3RS and hnRNP-U is maintained in vivo, we examined their subcellular localization by confocal microscopy. Nuclear co-

Figure 2. E3RS associates specifically and stoichiometrically with hnRNP-U. (A) 293 cells were transfected with Flag-E3RS (lane 1), Flag-WD (lane 2), Flag-HOS/ β -TrCP2 (lane 3), Flag-F-Box (lane 4), Flag-Smad-2 (lane 5), and HA-Skp2 (lane 6). The cells were lysed and the extracts immunoprecipitated, using agarose-conjugated anti-Flag (lanes 1–5) and anti-HA (lane 6) antibodies. Immunoprecipitated proteins were analyzed by SDS-PAGE and Coomassie brilliant blue staining. Exogenously expressed proteins are marked by asterisks. (B) The immunoprecipitated proteins from A were also analyzed by Western blot, using anti-Flag, anti-HA, and monoclonal anti-hnRNP-U antibodies. (C) Flag-tagged human and mouse hnRNP-U were cotransfected with HA-E3RS into 293 cells. Cell extracts were immunoprecipitated with anti-Flag antibodies, and precipitated proteins were analyzed by Western blot, using anti-HA and anti-Flag antibodies. (D) HeLa cells were transfected with Flag-WD or Flag-F-Box and stained using mouse anti-Flag and rhodamine-conjugated goat anti-mouse antibodies.

localization of the two molecules was observed upon co-expression of GFP-E3RS with Flag-hnRNP-U in 293 cells (Fig. 4A). On the other hand, Flag-hnRNP-U did not colocalize with nuclear GFP-histone 2A (Fig. 4B), showing the specificity of colocalization. In investigating the possible role of hnRNP-U in the subcellular localization of E3RS, we constructed several hnRNP-U mutants and examined their effect on the localization of GFP-E3RS in 293 cells. hnRNP-U carries a putative NLS motif at amino acid position 223–231 (Pinol-Roma et al. 1988; Kiledjian and Dreyfuss 1992). A two-amino-acid mutation (substitution of lysines 224 and 228 by alanine) within the NLS had no effect on the interaction of the mutant hnRNP-U (mNLS) with E3RS (data not shown), but resulted in mislocalization of the mutant protein to the cytoplasm (Fig. 4C). Another way of enforcing the cytoplasmic localization of hnRNP-U is through appending a nuclear export signal (NES) to the protein. The appended Rev-1-derived NES is responsible for exporting nuclear proteins to the cytoplasm via the Crm1 transport system (Henderson and Eleftheriou 2000). Coexpression of both mislocalized hnRNP-U proteins with GFP-E3RS in 293 cells resulted in the relocation of E3RS from the nucleus to the cytoplasm (Fig. 4C,E). Singly transfected cells, expressing only GFP-E3RS, retained the nuclear expression of E3RS, indicating that the driving force for expelling E3RS from the nucleus was its association with the mislocalized hnRNP-U. This effect is specific, because GFP-histone 2A localization was not affected by any of the hnRNP-U variants (Fig. 4D,F). To ascertain the fidelity of the NES-enforced cytoplasmic localization, NES-hnRNP-U (Fig. 4G) and mNLS-hnRNP-U (Fig. 4H) transfected cells were treated with Leptomycin B (LMB), an inhibitor of Crm1-dependent nuclear export, and analyzed for GFP-E3RS localization. LMB abolished the nuclear export of NES-hnRNP-U along with its associated GFP-E3RS (Fig. 4, cf. E and G), whereas it had no effect on the cytoplasmic localization of mNLS-hnRNP-U and GFP-E3RS (Fig. 4C,H).

To further confirm the *in vivo* association of E3RS and hnRNP-U, we examined the subcellular position of E3RS coexpressed with properly and mislocalized hnRNP-U fragments. The mislocalized, NLS-mutated N-terminal hnRNP-U fragment that binds E3RS (see Fig. 3B) redirected GFP-E3RS from the nucleus to the cytoplasm (Fig. 4J). On the other hand, a mislocalized C-terminal fragment, which does not bind E3RS (see Fig. 3B), had no effect on the nuclear position of E3RS (Fig. 4L). Thus, again, the subcellular localization of E3RS was altered only by the mutant hnRNP-U fragment that binds the E3 molecule.

Surprisingly, whereas NES-hnRNP-U forced the cytoplasmic localization of E3RS, cytoplasmic hnRNP-U localization was not observed upon expression of a NES-appended E3RS. NES-E3RS, in contrast to the wild-type protein, was present exclusively in the cytoplasm and was redirected to the nucleus upon LMB treatment (Fig. 5A). Nevertheless, the cytoplasmic position of NES-E3RS did not alter the nuclear localization of GFP-hnRNP-U (Fig. 5A), or of endogenous hnRNP-U (data not

Figure 3. E3RS binds the acidic N-terminal domain of hnRNP-U. (A) Schematic representation of hnRNP-U and its fragments N, N-198, and C. Indicated are the positions of various structural segments of the molecules (Pinol-Roma et al. 1988; Kiledjian and Dreyfuss 1992). Underlined are the NLS lysine residues, which, when substituted, compromise NLS function (see below). (B) 293 cells were transfected with Flag-tagged hnRNP-U or its single fragments (as in A; marked here with an asterisk), together with HA-E3RS. Cell lysates were prepared and immunoprecipitated using anti-Flag antibodies. Western blot analysis was performed using anti-Flag, HA, and hnRNP-U antibodies. Of note is the slower migration of the N-terminal fragments running at a position corresponding to ~1.5× their predicted size, possibly owing to a charge effect or a posttranslational modification at the N-terminal region.

shown). This was not caused by steric hindrance of the NES appendage, because NES-E3RS coimmunoprecipitated hnRNP-U, similarly to wild-type E3RS (data not shown). Taking into account the different localization of wild-type and NES-E3RS, we evaluated *in vivo* properties of the two E3RS forms. Overexpression of NES-E3RS had no significant effect on IκBα degradation or NF-κB activation (data not shown). On the other hand, there was a remarkable difference between the half-life of wild-type and that of NES-appended E3RS: pulse-chase analysis (Fig. 5B) indicated that the half-life of wild-type E3RS is ~120 min versus 20 min for NES-E3RS. LMB treatment reversed the shortened half-life of NES-E3RS (Fig. 5B), suggesting that either the nuclear position or the association with hnRNP-U was respon-

sible for the stability of E3RS. To determine which of these factors plays a major role in E3RS stability, the half-life of wild-type E3RS was examined while it was retained in the cytoplasm by mNLS-hnRNP-U (see Fig. 4C). Cotransfection of the two proteins resulted in significant stabilization of cytoplasmic wild-type E3RS compared with cytoplasmic NES-E3RS (Fig. 5B), indicating that association with hnRNP-U is probably the major stabilization factor.

The E3RS-hnRNP-U association resembles an E3-substrate interaction

The interaction of E3RS with its substrate is abrogated by a short phosphorylated peptide (pp10) representing

Figure 4. E3RS colocalizes with hnRNP-U in cell nuclei. (A,B) 293 cells were transfected with Flag-hnRNP-U (red) together with (A) GFP-E3RS (green) or (B) GFP-Histone 2A (green) and stained using anti-Flag and secondary Cy-5-conjugated antibodies. (C,D) Transfection with Flag-hnRNP-U-mNLS (red) together with (C) GFP-E3RS or (D) GFP-Histone 2A. Of the hnRNP-U-mNLS transfectants, 95% drove E3RS into the cytoplasm. (E,F) Transfection with Flag-NES-hnRNP-U (red) together with (E) GFP-E3RS or (F) GFP-Histone 2A. Of the NES-hnRNP-U transfectants, 95% drove E3RS into the cytoplasm. (G,H) LMB treatment (20 ng/mL) of the cotransfectants of GFP-E3RS and (G) Flag-NES-hnRNP-U or (H) Flag-hnRNP-U-mNLS. (I,J) Transfection with GFP-E3RS (green) together with (I) N-hnRNP-U (red) or (J) its NLS mutated form N-hnRNP-U mNLS (red). hnRNP-U fragments are as in Figure 3A. (K,L) Transfection with GFP-E3RS together with (K) C-hnRNP-U or (L) its NLS mutated form C-hnRNP-U mNLS.

Figure 5. hnRNP-U association affects E3RS stability. (A) 293 cells were transfected with Flag-NES-E3RS (red) together with GFP-hnRNP-U and stained with anti-Flag and secondary Cy-5-conjugated antibodies (left panel). Flag-NES-E3RS transfectants were treated for 1 h with (right panel) or without (middle panel) Leptomycin B (20 ng/mL). (B) Nuclear E3RS is stable compared with E3RS exported to the cytoplasm. 293 cells were transfected with wild-type E3RS alone or together with hnRNP-U-mNLS (upper strip) or NES-E3RS (lower strip), pulse-labeled with ³⁵S-methionine, and chased with unlabeled medium for the indicated intervals (in minutes). NES-E3RS was chased with or without Leptomycin B (20 ng/mL). Total cell extracts were prepared and analyzed by immunoprecipitation with anti-FLAG, SDS-PAGE, and phosphorimaging. E3RS signal quantitation is shown graphically.

the I κ B degradation motif, but not by an S/E-substituted I κ B α peptide (p10S/E; Yaron et al. 1997). To determine whether the E3RS-hnRNP-U association represents a receptor-ligand interaction, a complex composed of E3RS or its binding domain (F-box deleted E3RS, referred to as the WD-repeat fragment) and hnRNP-U was incubated with pp10. This peptide disrupted the interaction of hnRNP-U with E3RS (Fig. 6A, lane 2) or its WD fragment (Fig. 6A, lane 4), whereas p10S/E had no effect on the complex (Fig. 6A, lane 5). hnRNP-U was recovered in the pp10 eluate (Fig. 6A, lane 6), but not in the p10S/E eluate (Fig. 6A, lane 7). To test whether pp10 truly competes with hnRNP-U, we incubated complexes of E3RS/hnRNP-U and E3RS/pI κ B α with pp10 and measured the degree of displacement of hnRNP-U and pI κ B α from each complex, respectively (Fig. 6B). hnRNP-U was readily dissociated from E3RS, and a reduction of 50% occupancy was achieved at 3 μ M pp10. On the other hand, a much higher concentration of pp10 (600 μ M) was required to displace pI κ B α , indicating that the binding affinity of pI κ B α to E3RS is much higher than that of hnRNP-U. This and the nature of the displacement curves indicate a strictly competitive relationship between pI κ B α and hnRNP-U with respect to E3RS binding.

The specific association between the WD40-repeat domain of E3RS and hnRNP-U could resemble the interaction of the prototype WD-repeat structure of β -transducin with its partner, γ -transducin (Sondek et al. 1996). Several residues within the α -helix loops of β -transducin's first WD repeat contribute to this specific interaction (Lambright et al. 1996; Sondek et al. 1996). To test this possibility, we substituted through mutagenesis two E3RS lysine residues corresponding to similar residues of β -transducin that play a role in γ -transducin association. Mutagenesis was performed on the whole E3RS, or its

WD fragment. Although substitution of one of these lysine residues (WDK1, Lys 304) by alanine had no apparent effect on binding hnRNP-U by the WD fragment (Fig. 6C, lane 6), the substitution of Lys 326 (WDK2) abrogated hnRNP-U binding (Fig. 6C, lane 7). A similar, but less dramatic, effect of the K2 mutation was observed in hnRNP-U binding by the whole E3RS molecule (Fig. 6C, E3K2, lane 2). Interestingly, the effect of this mutation was corrected by saturating E3K2 with Skp1 (Fig. 6C, lane 4), thereby restoring hnRNP-U association to wild-type levels (Fig. 6C, cf. lanes 1,3,4).

We further examined the effect of the Lys mutants on binding pI κ B α (Fig. 6D). With the exception of WDK2, all of the mutants were indistinguishable from their wild-type counterparts in binding pI κ B α . pI κ B α binding by WDK2 was significantly, but not entirely, compromised, resulting in 10%–30% binding capacity. These results indicate that a single WD mutation has an inhibitory effect on the binding of both pI κ B α and hnRNP-U, particularly the latter, which can be compensated by the presence of an F-box, especially when Skp1 is added. We examined the subcellular localization of the E3RS mutants by immunofluorescence (Fig. 6E). Whereas WDK1 was located in the nucleus similarly to the wild-type WD fragment (see Fig. 2D), WDK2 was confined to the cytoplasm, probably owing to its failure to bind hnRNP-U.

Cumulatively, the peptide competition and mutation studies suggest that E3RS accommodates hnRNP-U and pI κ B α in a similar manner, yet with disparate affinities. Consequently, whereas the high affinity interaction results in the destruction of pI κ B, low affinity hnRNP-U is stable, both in vitro (Figs. 2, 6) and in vivo (Fig. 4) in the absence of proteasome inhibition. Attempts to ubiquitinate hnRNP-U in the presence of E3RS, E1, and several E2s (Ubc5C, UbcH7, and Ubc3) had negative results (data not shown; see the experiment in Fig. 7E). These obser-

ations suggest that hnRNP-U is a β -TrCP/E3RS ligand, but not a substrate, implying a pseudosubstrate interaction.

hnRNP-U engages an active, neddylated SCF ^{β -TrCP} complex and is exchanged by pIkB α prior to ubiquitination of the latter

The finding that E3RS binds hnRNP-U stably and stoichiometrically raised the question: how is it accessible for pIkB α ubiquitination? To examine the relationship of these three proteins, the hnRNP-U–E3RS complex was immobilized through E3RS or hnRNP-U and incubated with phosphorylated or nonphosphorylated I κ B α (Fig. 7A, lane 2), but not the nonphosphorylated I κ B α (Fig. 7A, lane 1). Yet, anchoring the same complex through hnRNP-U resulted in the failure to bind any I κ B α species (Fig. 7A, lanes 3 and 5). Instead, pIkB α (Fig. 7A, lane 6), but not I κ B α (Fig. 7A, lane 4), induced the dissociation of E3RS from the immobilized hnRNP-U into the post-slurry fraction. Coimmunoprecipitation of E3RS and pIkB α from the post-slurry fraction (Fig. 7A, lane 7) shows that they are in complex, indicating that the dissociation of E3RS from hnRNP-U was coupled to the binding of pIkB α .

Figure 6. The E3RS–hnRNP-U association resembles an E3–substrate interaction. (A) E3RS was anti-Flag-immunoprecipitated (lane 1), or immunoprecipitated and then incubated (lane 2) with a phosphorylated I κ B α peptide (pp10, containing the I κ B α degradation motif), washed, and analyzed by SDS-PAGE and Coomassie blue staining. The WD fragment was similarly immunoprecipitated in the absence (lane 3) or presence (lane 4) of pp10, or the serine-substituted peptide p10 S/E (lane 5), and the immune complexes were analyzed by SDS-PAGE and Coomassie staining. HnRNP-U was recovered in the WD pp10 eluate (lane 6) but not in the WD p10 S/E eluate (lane 7). (B) Displacement curves. 293 cells were transfected with Flag–E3RS together with Myc–SKP-1 to maximize hnRNP-U association (see Fig. 6C). Flag–E3RS/SKP1/hnRNP-U complexes were immobilized onto anti-Flag immunobeads, washed, and incubated with or without 35 S-methionine-labeled pIkB α . The amount of immobilized E3RS at each experimental point is depicted in the lower strip. Immunocomplexes were then subjected to increasing concentrations of the pp10 peptide and the dissociation of pIkB α (upper strip) or hnRNP-U (middle strip) per point was evaluated by autoradiography and Coomassie staining, respectively. The values of bound hnRNP-U and pIkB α at each point were entered into the displacement curves and processed by the Excel program. From the displacement curves it can be seen that the IC₅₀ of hnRNP-U (3 μ M) is much lower than that of pIkB α (600 μ M). (C) Flag-tagged E3RS and E3K2 were immunoprecipitated alone (lanes 1,2) or together with Myc–Skp1 (lanes 3,4) and analyzed by SDS-PAGE and Coomassie staining. Flag-tagged WD, WDK1, and WDK2 were similarly immunoprecipitated and analyzed (lanes 5–7). (D) The above extracts (panel C) were immunoprecipitated using anti-Flag immunobeads. The beads were incubated with 35 S pIkB α , washed extensively, and analyzed for pIkB binding. (E) Flag-tagged WDK1 and WDK2 were transfected into 293 cells, which were immunostained using anti-Flag antibodies and rhodamine-conjugated secondary antibodies. Fluorescence was visualized by confocal microscopy.

Figure 7. pIkBα displaces hnRNP-U from the SCF^{β-TrCP} complex and undergoes ubiquitination. (A) Mutually exclusive interaction of E3RS with hnRNP-U or pIkB. 293 cells transfected with Flag-E3RS were immunoprecipitated using anti-Flag (lanes 1,2,7) or anti-hnRNP-U (lanes 3–6) antibodies. IkBα (lanes 1,3) and IKK-phosphorylated IkBα (pIkBα; lanes 2,5) were incubated with the immunobeads, after which both the slurry (S; lanes 1–3,5) and the post-slurry fractions (PS; lanes 4,6) were analyzed by Western blot. Coimmunoprecipitation of E3RS and pIkBα from the post-slurry fraction in lane 6 (lane 7). (B) Endogenous Cul1 associates with endogenous hnRNP-U in the presence of endogenous nuclear E3RS. Nuclear and cytoplasmic fractions from HeLa cells were immunoprecipitated using goat anti-Cul1 or goat anti-p65 antibodies as a control. Western blot analysis was performed using anti-E3RS, anti-Cul1, and anti-hnRNP-U antibodies. In the input lanes, 10% of the immunoprecipitated lysates were applied to the gel. (C) hnRNP-U engages SCF^{β-TrCP} through E3RS. 293 cells were transfected with HA-Cul1 and Myc-Skp1 alone (lanes 1,4), or together with Flag-E3RS (lanes 2,5) or with Flag-E3RS alone (lanes 3,6). Cell lysates (5% of total input is shown) were immunoprecipitated with monoclonal anti-hnRNP-U antibodies (lanes 4–6) and analyzed by Western blot with the relevant antibodies. (D) Cul1 is preferentially neddylation in the hnRNP-U–SCF^{β-TrCP} complex. 293 cells were transfected with Flag-hnRNP-U together with (lanes 1,3) or without HA-E3RS (lanes 2,4). Total cell lysates were immunoprecipitated using anti-Flag antibodies. The precipitated endogenous Cul1 signal was compared to the input (10%) signal by Western blot. (E) The hnRNP-U–SCF^{β-TrCP} complex comprises a competent pIkBα E3 ligase. ³⁵S-labeled pIkBα was subjected to an immune-complex ubiquitin-ligase assay, using immunoprecipitated endogenous hnRNP-U as an E3 source (lanes 1,2; lane 2 employed 50% of the E3 source of lane 1). hnRNP-U immunoprecipitation from WD (dominant-negative; lane 3) or E3RS (lane 4) transfectants was used to show E3RS involvement. Immunoprecipitated hnRNP-U was subjected to the same assay, omitting pIkBα, without (lane 5) or with (lane 6) E1, and ubiquitination was monitored by Western blot analysis, using anti-hnRNP-U antibodies. (F) Schematic representation of the above data: (1) hnRNP-U occupies the neddylation SCF^{β-TrCP} complex via the E3RS WD region, but is not degraded (i.e., it is a pseudosubstrate). (2) In the presence of a true substrate (pIkBα), hnRNP-U dissociates and is replaced by pIkBα. (3) Unlike hnRNP-U, pIkBα is readily ubiquitinated and degraded, after which the SCF^{β-TrCP} complex may reassociate with its pseudosubstrate.

The specific and stable association of hnRNP-U with the E3RS WD domain should enable the engagement of other SCF components through the F-box domain. To test this assumption, SCF complexes were immunopurified from nuclear and cytoplasmic fractions with anti-Cul1 or control antibodies and analyzed by Western blot for their content of endogenous hnRNP-U and E3RS. Whereas similar amounts of Cul1 were detected in both fractions (Fig. 7B, lanes 3 and 4), both E3RS and hnRNP-U were detected only in the immunoprecipitates from the nuclear fraction (Fig. 7B, lane 4), despite the presence of hnRNP-U in both cellular fractions (Fig. 7B, lanes 1 and 6). Hence, it is likely that Cul1 associates with hnRNP-U only through the endogenous E3RS. Immunoprecipitation analysis of endogenous hnRNP-U from cells transfected with SCF components confirmed the specificity of hnRNP-U association with the SCF ^{β -TrCP}: whereas only minute amounts of Skp1 and Cul1 associated with hnRNP-U in the absence of exogenous E3RS, significantly higher levels of these components were precipitated upon E3RS overexpression (Fig. 7C, cf. lanes 4 and 5). It, therefore, appears that the SCF components interact with hnRNP-U via E3RS.

Previously, it was shown that the Cul1 component of a substrate-bound SCF is preferentially modified by the ubiquitin-related protein Nedd8, resulting in enhanced substrate ubiquitination (Tan et al. 1999; Read et al. 2000). We, therefore, examined the neddylation status of hnRNP-U-associated SCF ^{β -TrCP}. Cells were transfected with Flag-hnRNP-U, with or without HA-E3RS, and analyzed by Western blot for nedd8 modification of endogenous Cul1. Whereas most of Cul1 in the cell lysate is nonneddylated, hnRNP-U-associated Cul1 was found to be predominantly neddylated (Fig. 7D), provided that E3RS was included in the transfection. These results imply that hnRNP-U engages a fully assembled, neddylated E3 that is readily available for pIkB α ubiquitination. Indeed, immunoprecipitated endogenous hnRNP-U provided a source of highly efficient pIkB α -E3 activity. Incubation of the hnRNP-U immune complex with pIkB α , E1, and E2 (Ubc5C) resulted in a high level of pIkB α ubiquitination (Fig. 7E, lanes 1 and 2). Similar results were obtained using other cell sources (e.g., mouse embryonal fibroblasts and embryonic stem cells), showing the general nature of this observation. hnRNP-U-mediated pIkB α ubiquitination was E3RS-dependent, because overexpression of the dominant-negative F-box-deleted E3RS (WD, lane 3), but not of E3RS (Fig. 7E, lane 4), completely suppressed the reaction. To ascertain that hnRNP-U is not a true ubiquitin substrate, we subjected immunoprecipitated hnRNP-U to the same ubiquitination reaction, omitting pIkB α . In this experiment only a minute fraction of hnRNP-U undergoes mono- or diubiquitination, indicating that the associated E3, which efficiently ubiquitinates pIkB α , is virtually incapable of targeting hnRNP-U to ubiquitin-mediated degradation. The data pertaining to the relationship between SCF ^{β -TrCP} and the two ligands, hnRNP-U and pIkB α , are schematically represented (Fig. 7F).

Discussion

Our study was prompted by the surprising observation that E3RS resides predominantly in the nucleus of different cell types. This phenomenon has recently been reported for exogenously expressed β -TrCP/E3RS (Sadot et al. 2000; Lassot et al. 2001), yet our cell fractionation experiments confirm the nuclear position of the endogenous protein (Fig. 1). Being a component of an SCF complex, E3RS, which is itself devoid of a canonical nuclear localization signal, could be directed to the nucleus by an associated SCF component, such as Cul1/Roc1 (Furukawa et al. 2000). However, it appears that the cellular distribution of endogenous Cul1 and E3RS are quite distinct (Fig. 7B). Furthermore, only a small fraction of exogenously expressed E3RS is associated with endogenous Skp1 and Cul1 (data not shown), whereas nearly all of the overexpressed E3RS is in the nucleus. Therefore, the known SCF partners are unlikely to be responsible for the nuclear accumulation of E3RS. In our search for the E3RS transporter, we have identified a specific heterogeneous ribonucleoprotein, hnRNP-U, which is likely responsible for the nuclear localization of E3RS. hnRNPs are a diverse group of proteins containing RNA-binding motifs, which participate in multiple regulatory processes involving RNA and RNPs. Among the latter is mRNA splicing and transport, transcription, translation, and DNA recombination, the maintenance of telomere length, and the control of RNA stability (Krecic and Swanson 1999). Many of the 20 major hnRNPs shuttle in and out of the nucleus, yet reside predominantly in the nucleus (Nakielnny and Dreyfuss 1999). Although several shuttling hnRNPs are involved in mRNA transport, none has been implicated in protein transport to or from the nucleus. hnRNP-U is an abundant protein, which, similarly to many other hnRNPs, may participate in the maintenance of the internal nuclear architecture (Gohring et al. 1997). It adheres to the nuclear scaffold at A/T-rich regions through a specific DNA-binding domain (and is, therefore, also termed Scaffold Attachment Factor-A; Romig et al. 1992) and binds RNA through a separate domain, the RGG box (Kiledjian and Dreyfuss 1992). However, the *in vivo* role of this protein has not been resolved (Krecic and Swanson 1999).

hnRNP-U interacts selectively with β -TrCP/E3RS, as indicated by its failure to associate with its most closely related F-box protein β -TrCP2/HOS (Fig. 2), which also functions as an I κ B E3 (Fuchs et al. 1999), although significantly less effectively (data not shown). This selectivity is striking because β -TrCP2 displays 85% overall similarity to E3RS and 93% identity throughout the WD40-binding domain. Another mammalian F-box protein, Skp2, which contains a leucine-rich interaction domain, rather than the WD domain (Krek 1998), is also incapable of associating with hnRNP-U. Therefore, hnRNP-U is unlikely to be yet another common component of the SCF complex, and, as such, has no direct role in the ubiquitination process (Fig. 7). An intriguing feature of the E3RS/hnRNP-U association is the nature of their interaction. Coomassie staining (Figs. 2,6) shows

that these two proteins, but no other ones, undergo a stoichiometric interaction, indicating a direct association. E3RS/hnRNP-U binding breaks apart upon inclusion of a peptide representing the IκBα degradation motif (Fig. 6A), or pIκBα itself (Fig. 7A), where pIκBα functions as a strictly competitive inhibitor (Fig. 6B). An E3RS substrate is recognized by virtue of its IκB degradation motif (Yaron et al. 1997); its template DSGxxS is contained within hundreds of different proteins in the databanks (M. Davis and Y. Ben-Neriah, unpubl.). hnRNP-U does not harbor this motif, but could possibly be tethered to E3RS through one of several SxxxS sequences, provided it is doubly phosphorylated. This would be compatible with the finding that hnRNP-U is a low-affinity substrate of E3RS (Fig. 6B) and, in analogy to the stability of suboptimally phosphorylated Sic1 (Nash et al. 2001), could explain why hnRNP-U is a poor ubiquitination substrate.

Alternatively, E3RS could associate with hnRNP-U via charge-based interactions. We have identified the E3RS-interaction domain in hnRNP-U as the N-terminal acidic and glutamine-rich segment, wherein ~30% of the residues are aspartic or glutamic acids. Supporting this assumption is the loss-of-function phenotype of an E3RS mutation (K2, Fig. 6C), where a specific lysine residue is substituted in the first WD40 repeat of E3RS. An analogous residue occupies a position critical to the interaction of the β-transducin WD domain with its partner, γ-transducin (Lambright et al. 1996). The K2 mutant of the WD-repeat fragment showed defective binding of both pIκBα and hnRNP-U, although the effect was more pronounced in the binding of hnRNP-U. This is in contrast to mutation of K1, the homolog of another contact residue in the β-γ-transducin interface (Lambright et al. 1996), which had no influence on the binding of pIκBα or hnRNP-U. The combined effect of the K2 mutation reinforces the assumption that the two competitive substrates, pIκBα and hnRNP-U, occupy a similar or an overlapping binding site. Furthermore, the finding that the WDK2 mutant is exclusively cytoplasmic strengthens the hypothesis that hnRNP-U binding is needed for E3RS nuclear localization. Remarkably, the K2 deficiency was compensated to a large extent by the function of the F-box in the intact molecule E3K2. Thus, E3K2 is fully capable of binding pIκBα, and when saturated with Skp1, retains the capacity of wild-type E3RS for stoichiometric association with hnRNP-U. This result emphasizes a previous observation, regarding the role of Skp1 in the SCF complex (Bai et al. 1996; Feldman et al. 1997; Skowrya et al. 1997). Accordingly, Skp1 is more than an adapter between the F-box protein and the rest of the SCF (Cul-1, Roc1/Hrt1/Rbx1). It has a role in stabilizing the interaction of the F-box protein with the substrate, a property also supported by the recently reported structure of the Skp2/Skp1 complex (Schulman et al. 2000). Although the Skp1 stabilizing effect is mainly apparent in the case of a mutation that compromises a low-affinity binding ligand, it may be important for physiological interactions in vivo. As Skp1-deficient cells in all likelihood cannot be generated, it is impossible to evaluate at

this stage the complete phenotype of the K2 mutation in conjunction with an intact F-box protein.

Is hnRNP-U a true substrate? Probably not, for several reasons: (1) the abundance of hnRNP-U in the cell would prohibit effective substrate degradation; (2) the stoichiometric association of hnRNP-U with E3RS is observed in the absence of proteasome inhibition and is, therefore, nonproductive with respect to degradation; (3) hnRNP-U is poorly ubiquitinated under conditions promoting efficient pIκBα ubiquitination. Thus, hnRNP-U is more likely a pseudosubstrate, occupying the substrate-binding site of the E3 receptor, but avoiding the common fate of ubiquitin substrates. In this respect, it may serve as a safety plug, prohibiting the engagement of the E3 with inappropriate substrates, including ones harboring a sequence similar to the IκB degradation motif (Karin and Ben-Neriah 2000).

As a pseudosubstrate, hnRNP-U is capable of stably associating with a functional SCF complex, as reflected by the coimmunoprecipitation of E3RS, Skp1 and Cul-1 by hnRNP-U-specific antibody or the coimmunoprecipitation of hnRNP-U and E3RS by anti-Cul1 antibodies (Fig. 7B,C). Moreover, while occupied by hnRNP-U, similarly to other substrate-engaged SCFs, the associated Cul-1 is largely modified by Nedd-8 (Fig. 7D), an apparent prerequisite for efficient pIκBα ubiquitination (Furukawa et al. 2000; Read et al. 2000). As such, immunoprecipitation of endogenous hnRNP-U provides a ready source of specific E3 from different cells and tissues. Upon engaging a bona fide substrate, such as pIκBα, the SCF^{β-TrCP} complex readily dissociates from hnRNP-U and is capable of inducing an efficient ubiquitination reaction (Fig. 7A,E). We verified this property by immunoprecipitating an active endogenous pIκBα-E3 via hnRNP-U from diverse cell types, (e.g., mouse embryonal fibroblasts and embryonic stem cells; data not shown). We assume that a similar process occurs in vivo, provided that the affinity of the candidate substrate is sufficiently high to allow it to compete with hnRNP-U for the binding of β-TrCP/E3RS. Accordingly, SCF^{β-TrCP} would be continuously occupied by a pseudo- or true substrate (see Fig. 7F), thereby guaranteeing the stability and preparedness (full SCF assembly and neddylation) of the E3. An obvious advantage of the hnRNP-U pull-down E3 assay is the possibility to specifically monitor the activity of SCF^{β-TrCP} in different physiological and pharmacological settings.

As a stoichiometric E3RS partner, hnRNP-U appears a likely candidate for conveying E3RS into the nucleus or retaining it there in case it has entered the nucleus via another route. Cell fraction analysis and confocal microscopy of E3RS shows that the bulk of the protein is found in the nucleus (Fig. 1), where it is colocalized with hnRNP-U (Fig. 4). Colocalization of the two molecules is preserved upon enforcing the cytoplasmic localization of hnRNP-U, either through an NLS mutation or by appending a nuclear export signal (NES) to hnRNP-U (Fig. 4C,E). This attests to the maintenance of the tight interaction between the two molecules in vivo. Interestingly, the cytoplasmic expression of NES-E3RS was not ac-

accompanied by relocalization of hnRNP-U to the cytoplasm, resulting in the segregation of the two proteins (Fig. 5A). The reason for the disparity between the effects of the two NES-appended molecules is seemingly unclear. It is likely caused by hnRNP-U retention by the nuclear matrix, which can overcome NES in *trans*, but not in *cis*. While segregated from its pseudosubstrate, the half-life of NES-E3RS is significantly shorter than that of the wild-type protein, but the extended half-life is restored upon relocation of the protein to the nucleus by LMB treatment (Fig. 5B). However, it appears that the stabilization of E3RS is mainly due to its interaction with hnRNP-U, rather than to its localization per se (Fig. 5B), alluding to an additional role of the E3RS-hnRNP-U interaction: stabilization of the F-box protein. F-box proteins are often unstable, possibly because of autoubiquitination (Zhou and Howley 1998; Galan and Peter 1999). Indeed, protection of an F-box protein from self-destruction via engagement of a substrate has previously been proposed (Deshaies 1999) and is supported by the study of the Skp2/Skp1 complex, showing that in the absence of a substrate, the tail of the F-box protein occupies its putative substrate-interaction site (Schulman et al. 2000).

Considering the cytoplasmic position of the known β -TrCP/E3RS substrates (Maniatis 1999), the finding that the F-box protein is predominantly nuclear is enigmatic. Conceivably, hnRNP-U may shuttle in and out of the nucleus like other hnRNPs, cotransporting E3RS as with the NES-appended hnRNP-U (Fig. 4). The minor cytoplasmically exported E3RS fraction could then constitute the functional E3, whereas the major nuclear pool would serve as a depot for its replenishment. On the other hand, there is ample evidence that the cellular position of I κ B is not static, but the result of a dynamic process set by opposing forces: (1) a p50 nuclear localization signal (NLS), which is not concealed by I κ B α , therefore directing the I κ B α /NF- κ B complex into the nucleus (Huxford et al. 1998; Jacobs and Harrison 1998); (2) an unidentified transporter associated with the I κ B α ankyrin repeats, channeling newly synthesized I κ B α into the nucleus (Turpin et al. 1999); (3) potent nuclear export signals within I κ B α (Arenzana-Seisdedos et al. 1997; Johnson et al. 1999; Tam and Sen 2001), forcing the complex out of the nucleus. This dynamic process could then be interrupted by I κ B α ubiquitination and degradation, either in the nucleus or outside, resulting in the unopposed nuclear localization of NF- κ B through both the NLS of p50 and p65. Nuclear ubiquitination, which is a well-documented event (e.g., Far1 ubiquitination by cdc4 [Blondel et al. 2000] or p53 ubiquitination by Mdm2 [Yu et al. 2000]), might complement NF- κ B activation by eliminating the DNA-binding inhibitory effect of pI κ B α that escaped cytoplasmic degradation. In support of this model, both β -TrCP/E3RS-dependent nuclear degradation of pI κ B α and subsequent NF- κ B activation were observed in leptomycin B-treated cells, where nuclear export of I κ B α was inhibited by the drug (Johnson et al. 1999; Renard et al. 2000). Interestingly, hnRNP-U was reported to undergo cleavage in apoptotic cells (Gohring

et al. 1997), a process that could result in its dissociation from E3RS. A possible consequence would be the compromise of E3RS function and NF- κ B activation, thus augmenting apoptosis (Karin and Ben-Neriah 2000). Nonetheless, E3RS could lurk in its nuclear base, targeting nuclear substrates as well. The A/T-rich scaffold-associated DNA sites, which tether hnRNP-U (Romig et al. 1992), serve as binding sites for other important regulatory proteins, some of which may carry the I κ B α degradation motif (e.g., CREB family proteins; Taylor et al. 2000; Lassot et al. 2001). Thus, E3RS could reach a variety of nuclear proteins whose expression is regulated posttranslationally by the ubiquitin-proteasome system.

Materials and methods

Expression vectors and antibodies

hnRNP-U was PCR-cloned from both human and mouse cDNA libraries into the pFLAG-CMV-2 expression vector (Kodak) at the *NotI* site. The *NotI* cleavage site was incorporated into both the forward primer, 5'-tagcgccgcgaatgagttcctgcctgtt-3', and the reverse primer, 5'-tagcgccgcgctcaataatccttggtgata-3'. The expression vectors encoding Flag-tagged E3RS/ β -TrCP (Flag-E3RS), the WD fragment (F-box-deleted E3RS), and the F-box fragment (Flag-F-box) have been described (Yaron et al. 1998). The REV1 NES was attached to the C terminus of Flag-hnRNP-U and the N terminus of Flag-E3RS by PCR using four primers: 5'-cccttagagcgtttaactctagactgcaacgaggaattccgccacgc-3' and 5'-agcctc taaggaggcaactgaagtgggacgggaagcttcgggcccgc-3', together reconstituting the NES sequence, and the flanking primers 5'-cgggc ccgaagctt-3' and 5'-gctgtgcggaattc-3' were used for inserting the NES fragment in frame. The hnRNP-U fragments N (amino acids 1–407), N-198 (amino acids 1–198) were prepared by digesting the Flag-hnRNP-U vector with *BglII* and *KasI*, respectively, followed by religation. C-hnRNP-U (amino acids 201–806) was prepared by digestion with *SmaI* and insertion into the Flag vector at the *NotI* site. Lysines 224 and 228 of the hnRNP-U NLS were converted to alanine, using the complementary primers: 5'-ataaagcaaggggtgttgaaga-3' and 5'-tcttgcaa cacccttgctttat-3'. HA-E3RS was prepared by subcloning human E3RS into the pCGN-HA vector. Mutagenesis of Lys 304 (K1) and Lys 326 (K2) residues to Ala in the Flag-WD construct was performed using the Quickchange kit (Stratagene). These were designated WDK1 and WDK2, respectively. The K2 residue in Flag-E3RS was similarly mutated to Ala (E3K2). Human *HOS*/ β -TrCP2 cDNA was obtained by PCR-cloning according to GenBank entry AB033281 into the pFLAG-CMV-2 expression vector (Flag-HOS/ β -TrCP2). HA-Cul1, HA-Skp2, and Myc-Skp1 expression plasmids (Lyapina et al. 1998) were provided by R.J. Deshaies (Caltech, Pasadena, CA) and the GFP-Histone 2A vector was provided by M. Brandeis (Hebrew University Jerusalem, Israel). The Flag-Smad 2 expression vector (Wu et al. 2000) was used as a negative control. The MEKK1 and IKK2 mammalian expression plasmids were described before (Mercurio et al. 1997; Yin et al. 1998).

Agarose-conjugated (A-1205) and purified monoclonal anti-Flag M2 antibodies (F-3165) were purchased from Sigma. Monoclonal anti-hnRNP-U antibodies (3G6; Pinol-Roma et al. 1988) were provided by G. Dreyfuss (University of Pennsylvania, Philadelphia). Sepharose-immobilized anti-p65 (anti-NF- κ B; sc-109 AC), anti-HA (sc-805), polyclonal goat anti-E3RS serum (sc#8863) and goat anti-Cul1 (sc#8552) were from Santa Cruz.

The rabbit polyclonal anti-IκBα antibody was described in Alkalay et al. (1995). Mouse monoclonal anti-Myc antibodies, c-myc (Ab-1), were purchased from Oncogene Research Products. The goat anti-mouse RedX and goat anti-mouse Cy5 fluorescent secondary antibodies were purchased from Jackson ImmunoResearch (115-295-062, 115-175-146).

Transfections and immunofluorescence

CHO, HeLa, or 293T cells were plated on gelatin-coated plates, and transfections were performed using FuGENE 6 (Roche) or calcium phosphate. For immunofluorescence, cells were plated on gelatin-coated cover slips, and immunostaining was performed at 24 h posttransfection. The cells were washed with phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde in PBS for 30 min at 4°C, permeabilized for 6 min using 0.25% Triton X-100 in PBS, and washed with PBS. The cover slips were blocked for 45 min with 4% bovine serum albumin in 0.1% PBS-Tween 20. Primary antibodies anti-Flag (1:600) and anti-HA (1:100) diluted in 4% bovine serum albumin, PBS-Tween-20 were added to the cover slips and incubated for 60 min. The cover slips were washed 3× with PBS, and then incubated with secondary fluorescent antibodies, goat anti-mouse RedX (1:150) for 45 min. Following three washes with PBS, the cover slips were mounted on slides and analyzed by confocal microscopy. For pulse-chase analysis, transfected 293T cells were incubated for 1 h in medium lacking methionine, then pulsed with 40 μCi/mL of [³⁵S]methionine for 25 min, after which they were washed twice with PBS and chased either with or without LMB (20 ng/mL). Whole-cell extracts (125 μg) were immunoprecipitated with anti-Flag and analyzed by PhosphorImaging.

Immunoprecipitation and immunopurification

Cells were harvested 24–48 h after transfection and extracted by suspending in 50 mM Tris (pH 7.6), 1 mM dithiothreitol (DTT), 0.1% Nonidet P-40 (NP-40), 1 mM phenyl methyl sulfonyl fluoride, and Aprotinin (10 μg/mL) and vortexing for 20 sec. Cell extracts were collected following centrifugation at 20,000g at 4°C for 30 min. Prior to immunoprecipitation 150 mM NaCl was added to the cell extract. For Coomassie blue staining, Flag-tagged proteins were immunoprecipitated from 1 mg of lysate of 293T transfected cells with 5 μL of the anti-Flag immunobeads. Immunoprecipitation was carried out at 4°C for 2 h, and the immunobeads were washed 4× in 300 mM NaCl, 1 mM DTT, 0.1% NP-40, and 50 mM Tris (pH 7.6). Flag-tagged proteins were eluted from the Flag immunobeads with 1 mg/mL of Flag peptide in 50 mM Tris, 1 mM DTT (pH 7.6) at 25°C for 30 min. hnRNP-U was immunoprecipitated, using 0.2–0.5 μL of the 3G6 antibody and 2–5 μL of Protein G immunobeads. The Flag-E3RS or the Flag-WD/hnRNP-U complex was disrupted by adding the phosphorylated IκB peptide pp10 (Yaron et al. 1998) to the extract at a concentration of 1 mg/mL. E3RS-associated proteins were eluted from the washed Flag immunobeads by incubating them with pp10 (1 mg/mL) at room temperature for 30 min. Control elutions were performed with the serine-substituted IκBα peptide p10 S/E under similar conditions.

IκB ubiquitination and binding assay

Endogenous hnRNP-U from 293T cells was used as an E3 source. 100 μg of protein extract was immunoprecipitated using 0.2 μL of anti-hnRNP-U antibodies and protein G Sepharose. IκB was phosphorylated by the constitutively active IκB kinase (IKK-2E; Mercurio et al. 1997) and subjected to binding and

ubiquitination assays, as described previously (Yaron et al. 1998).

Protein identification

Proteins were identified by mass spectrometry as previously described (Shevchenko et al. 1996). Briefly, gel bands were excised from a one-dimensional gel stained with Coomassie colloidal blue and digested in-gel with trypsin. The recovered unseparated peptide mixture was analyzed by MALDI mass spectrometry, using a Bruker Reflex III MALDI time-of-flight mass spectrometer (Bruker Daltronics). Samples for MS/MS analysis were prepared essentially as described. After in-gel digestion, the supernatant was loaded onto a Poros R2 (Perseptive Biosystems) microcartridge (Wilm et al. 1996) and eluted into nanoelectrospray needles (Protana, Odense). Nanoelectrospray MS/MS analysis was performed on a QSTAR quadrupole time-of-flight mass spectrometer (Perkin Elmer-Sciex), and fragmentation spectra were obtained for as many peptides as possible. PepSea software (Protana) was used to search publicly available sequence databases maintained by NCBI with a list of peptide masses or with peptide sequence tags from fragmentation spectra.

Acknowledgments

We thank R.J. Deshaies, M. Brandeis, and G. Dreyfuss for providing invaluable plasmids and antibodies; M. Tarshish for help with the confocal microscope; and I. Alkalay for comments on the manuscript. This research was supported by grants from the Israel Science Foundation funded by the Israel Academy for Sciences and Humanities–Centers of Excellence Program, the German-Israeli Program (DIP), and Boehringer Ingelheim International. Work at the Protein Interaction Laboratory was supported by a grant from the Danish National Research Foundation to the Centre for Experimental Bioinformatics.

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Pseudosubstrate regulation of the SCF^β-TrCP ubiquitin ligase by hnRNP-U

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Genes Dev. 2002, **16**:

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