A Quality Control Pathway That Down-regulates Aberrant T-cell Receptor (TCR) Transcripts by a Mechanism Requiring UPF2 and Translation*

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The nonsense-mediated decay (NMD)1 pathway is a RNA surveillance pathway that degrades mRNAs containing premature termination codons (PTCs). T-cell receptor (TCR) and immunoglobulin (Ig) transcripts, which are encoded by genes that very frequently acquire PTCs during lymphoid ontogeny, are down-regulated much more dramatically in response to PTCs than are other known transcripts. An additional feature unique to TCR, Ig, and a subset of other mRNAs is that they are down-regulated in response to nonsense codons in the nuclear fraction of cells. This is paradoxical, as the only well recognized entity that recognizes nonsense codons is the cytoplasmic translation apparatus. Therefore, we investigated whether translation is responsible for this nuclear-associated mechanism. We found that the down-regulation of TCR-β transcripts in response to nonsense codons requires several features of translation, including an initiator ATG and the ability to scan. We also found that optimal down-regulation depends on a Kozak consensus sequence surrounding the initiator ATG and that it can be initiated by an internal ribosome entry site, neither of which has been demonstrated before for any other PTC-bearing mRNA. At least a portion of this down-regulatory response is mediated by the NMD pathway as antisense hUPF2 transcripts increased the levels of PTC-bearing TCR-β transcripts in the nuclear fraction of cells. We conclude that a hUPF2-dependent RNA surveillance pathway with translation-like features operating in the nuclear fraction of cells prevents the expression of potentially deleterious truncated proteins encoded by non-productively rearranged TCR genes.

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1 The abbreviations used are: NMD, nonsense-mediated decay; PTC, premature termination codon; TCR, T-cell receptor; IRES, internal ribosome entry site; hUPF2, human UPF2; UTR, untranslated region; RPA, RNase protection analysis.
translation, including the ability to be down-regulated by an internal ribosome entry site (IRES), a cis element that specifically recruits ribosomes for translation (22). Lastly, to determine whether TCR-β down-regulation in response to nonsense codons has features of classic NMD, we examined the role of UPF2, which has been shown in S. cerevisiae to be required for NMD. Our antisense studies clearly showed that the down-regulation of TCR-β transcripts depends on human UPF2 (hUPF2). To our knowledge, this is the first time that hUPF2 has been shown to be required for the down-regulation of any transcript in response to nonsense codons in mammalian cells.

EXPERIMENTAL PROCEDURES

Plasmids—Construct A (β-290) contains a wild-type TCR-β gene with a full-length open reading frame (pAcO/IF in Ref. 16); B and C (β-367, β-368, respectively) are derivatives of A that contain nonsense (TAA) and missense (TAC) mutations at codon 68 in the VDJ exon generated by site-specific mutagenesis, respectively. D, E, and F (β-658, β-627, and β-617, respectively) each contain a stem loop (ΔG ~ 61 kcal/mol) identical to that previously shown to impede translation (23) at a site 42 nt upstream of the initiating ATG in constructs B, C, and A, respectively. G (β-495) is a TCR-β minigene (containing L, VDJ, and a C2.1-C2.4 chimeric exon) cloned between the Sall and HindIII sites of the vector pBS-C-2-C4 (EV 147) (24). The TCR-β minigene in G is identical to the one we previously described (construct C in Ref. 19) except that the JC intron was shortened from 1021 nt to 358 nt by cutting out an internal Eco 0191 fragment. H (β-496) is identical to G except that it has a TAA nonsense codon at codon 68. I (β-497) and J (β-497) were prepared by inserting a 1.1-kb ClaI/NotI fragment from pSBC-1-C2 (EV 147), which contains the type I poliovirus IRES (nt 1–628 of the 5′-untranslated region (UTR)) (24) between the ClaI and NotI sites of G and H, respectively. K (β-600) is identical to B except that it has mutated initiator ATGs rendered defective as previously described (construct I in Ref. 19). L (β-639) is a derivative of B that has only the VDJ exon initiating ATG mutated as previously described (construct H in Ref. 19). M, N, O, and P (β-626, β-638, β-647, and β-665, respectively) are derivatives of construct L that contain point mutations in the Kozak consensus sequences. Q (β-760) and R (β-756) are identical to N and P, respectively, except that they lack PTCs. S (β-595) is a derivative of A that contains a nonsense mutation (TAG) at codon 98 (in the VDJ exon). The anti-UPF2 construct (G-407) was generated by inserting a 0.5-kb hUPF2 fragment between the Sall and BsmHI sites of the pHpApr-1 vector (20) such that it is in the antisense orientation with respect to the β-actin promoter. The hUPF2 fragment was a PCR product generated with the primers MDA-720 (5′-CTGGGATCCCGA-GCCGCTAGTTGTGC-3′) and MDA-703 (5′-CCGTACGCGTGAATGATCTC-3′) using the plasmid pCMV-rent 2 (G-314) (25) as the template. G-1F was prepared by inserting the human β-globin gene into the DraI and BglII sites of EV-107. All mutations introduced in the constructs described above were generated by site-specific mutagenesis (27).

Transfection, RNA Isolation, and RNase Protection Analysis—DNA constructs were transiently transfected into HeLa cells using LipofectAMINE according to the manufacturer’s instructions (Invitrogen). Total and nuclear RNA were isolated as described previously (28). The TCR-β mRNA levels were determined using a direct radioactivity scanner (Instant Imager; Packard Instruments, Downers Grove, IL). RNase protection analysis (RPA) and the riboprobes (TCR-β, neomycin, β-actin) used for this analysis were described previously (28). The β-globin riboprobe template is a 250-nt PCR fragment containing 50 nt of the 3′ end of human β-globin intron 2 and 200 nt of exon 3. The hUPF2 riboprobe template is a 189-nt hUPF2 3′-cDNA fragment. We determined that our RPA assay was quantitative by performing titration experiments; increasing the amount of input RNA linearly increased the level of the protected TCR-β bands, whereas increasing the amount of riboprobe had no effect on the protected bands, indicating that excess probe was present in the annealing reaction (data not shown).

RESULTS

A Stem Loop Reverses TCR-β Down-regulation in Response to a Nonsense Codon—To examine the role of translation in the down-regulation of TCR-β mRNA in response to nonsense codons, we transiently transfected TCR-β constructs into HeLa cells. We used HeLa cells because they reproduce all aspects of the PTC-mediated down-regulation of TCR-β transcripts that we have observed in stably transfected T cells (16, 18, 28). A major advantage of HeLa cells over T cells is that they can be transiently transfected with sufficient efficiency to permit analysis of the RNA products by RPA.

To determine whether the down-regulation of TCR-β transcripts in response to nonsense codons depends on a scanning process, we introduced a stem loop in the 5′-UTR of a functionally rearranged Vα1-Dα1-Jα2 gene (labeled Lα and Dα2, respectively) and four C region exons (labeled Cα2, Cα2, Cα2, and Cα2). Constructs B and C contain nonsense and missense mutations, respectively, at codon 68 but otherwise are identical to construct A. Constructs D, E, and F are identical to B, C, and A, respectively, except that they contain a stem loop in the 5′-UTR. Because all constructs also contain an independent transcription unit encoding neomycin, neomycin (Neo) mRNA levels were used as a measure of transfection efficiency. The TCR-β mRNA band protected by the TCR-β probe is ~72 nt, which is the size expected based on the positions of the splice sites in TCR-β mRNA. Similar results were obtained in three independent transfection experiments.

FIG. 1. A stem loop reverses TCR-β down-regulation in response to a nonsense codon. RPA of total cellular RNA (10 μg) isolated from HeLa cells transiently transfected with the constructs is shown. Construct A consists of the L and VDJ exons of a functionally rearranged Vα1-Dα1-Jα2 gene (labeled Lα and Dα2, respectively) and four C region exons (labeled Cα2, Cα2, Cα2, and Cα2). Constructs B and C contain nonsense and missense mutations, respectively, at codon 68 but otherwise are identical to construct A. Constructs D, E, and F are identical to B, C, and A, respectively, except that they contain a stem loop in the 5′-UTR. Because all constructs also contain an independent transcription unit encoding neomycin, neomycin (Neo) mRNA levels were used as a measure of transfection efficiency. The TCR-β mRNA band protected by the TCR-β probe is ~72 nt, which is the size expected based on the positions of the splice sites in TCR-β mRNA. Similar results were obtained in three independent transfection experiments.

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FIG. 2. TCR-β mRNA down-regulation in response to a nonsense codon can be elicited by an internal ribosome entry site (IRES). RPA of total cellular RNA (10 μg) isolated from HeLa cells transiently transfected with the constructs is shown. Construct G is a TCR-β minigene composed of three exons, including a chimeric C exon (labeled Cβ) that has portions of the Cα22-1 and Cα22-4 exons, driven by an MT7 retroviral promoter (24). Construct H is identical to G except that an IRES was inserted before the ATG start codon. A plasmid expressing the β-globin gene (G1-F) was cotransfected with the TCR-β constructs to permit measurement of transfection efficiency. Similar results were obtained in at least three independent transfection experiments.

FIG. 3. An initiator ATG and surrounding Kozak consensus nucleotides are essential for optimal down-regulation of TCR-β transcripts in response to a nonsense codon. RPA of total cellular RNA (10 μg) isolated from HeLa cells transiently transfected with the constructs is shown. mRNA levels were quantitated as described in Fig. 1. Similar results were obtained in at least three independent transfection experiments. A, mutation of both initiator ATGs reversed the down-regulation of TCR-β transcripts bearing a PTC. The ATG in the L exon is the normal translation start site; the ATG in the VDJ exon is in the same reading frame as the L exon start codon. Construct H has both ATGs mutated and has a PTC at codon 68. B, the Kozak consensus sequence around the initiator ATG is required for optimal down-regulation in response to a nonsense codon. Constructs N and P have a PTC at codon 68, whereas constructs A, Q, and R have the normal sense codon at this position. All constructs have a normal L exon ATG and a mutated VDJ exon ATG. The sequences surrounding the L exon ATG in each construct are indicated in Table I.
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The Kozak consensus sequence (R \( \rightarrow \) A, G) and G are the most critical residues for start-site recognition. The VDJ exon ATG in each construct was disrupted by mutation, whereas the ATG in the L exon (shown) is intact. mRNA levels were determined by RPA and normalization against neomycin mRNA levels (as in Fig. 1). The values obtained reflect the average and standard error from three to four experiments.

| Construct | L exon mRNA level | ATG | TAG | TGA |
|-----------|------------------|-----|-----|-----|
| L         | 1.00             | S   | A   | S   |
| M         | 0.13 ± 0.20      |     |     |     |
| N         | 0.64 ± 0.18      |     |     |     |
| O         | 1.09 ± 0.03      |     |     |     |
| P         | 1.67 ± 0.15      |     |     |     |

The NMD Factor UPF2 Plays a Role in the Down-regulation of TCR-\( \beta \) Transcripts in the Nuclear Fraction of Cells—Once we had obtained several lines of evidence that translation is required for PTC-induced down-regulation of TCR-\( \beta \) transcripts, we next assessed whether this down-regulation is exerted using the NMD RNA surveillance pathway. We considered the possibility that TCR and Ig transcripts use a pathway distinct from NMD, as these mRNAs are transcribed from genes that acquire PTCs much more frequently than do other genes, are down-regulated more strongly in response to nonsense codons than are other known transcripts, and may require a unique second signal to be down-regulated (29, 30). To examine the role of NMD, we assessed whether TCR-\( \beta \) down-regulation required hUPF2. Although human UPF2 has not been proven to be involved in mammalian NMD, its orthologues in S. cerevisiae (Upf2) and Caenorhabditis elegans (Smg3) have been shown to be essential for NMD (3, 6, 7). Furthermore, tethering a hUPF2/MS2 fusion protein downstream of the stop codon in \( \beta \)-globin mRNA triggers an NMD-like response in HeLa cells (34).

To determine the role of hUPF2 in TCR-\( \beta \) down-regulation, we generated an expression plasmid that transcribes antisense hUPF2 mRNA. This anti-hUPF2 plasmid was cotransfected with TCR-\( \beta \) plasmids into HeLa cells, followed by RPA of nuclear RNA from these cells. Cotransfection of the anti-hUPF2 plasmid partially reversed the down-regulation of nonsense constructs. To determine whether the anti-hUPF2 plasmid was cotransfected with either the anti-hUPF2 plasmid or a control vector-only plasmid. Similar results were obtained in at least two independent transfection experiments.

**FIG. 4.** hUPF2 plays a role in nonsense codon-induced down-regulation of TCR-\( \beta \) transcripts in the nuclear fraction of cells. This anti-hUPF2 probe is \( \sim \)190 nt. hUPF2 mRNA levels were determined by normalizing against the level of endogenous \( \beta \)-actin transcripts. Constructs A and S were cotransfected with the constructs shown, B, antisense hUPF2 decreases the level of endogenous hUPF2 mRNA. The hUPF2 mRNA band protected by the hUPF2 probe is \( \sim \)190 nt. hUPF2 mRNA levels were determined by normalizing against the level of endogenous \( \beta \)-actin transcripts. Constructs A and S were cotransfected with either the anti-hUPF2 plasmid or a control vector-only plasmid. Similar results were obtained in at least two independent transfection experiments.
nous hUPF2 mRNA (~3-fold) compared with its level after cotransfection with a control vector-only plasmid (Fig. 4B). We therefore conclude that hUPF2 participates in the down-regulation of TCR-β transcripts in response to nonsense codons. To our knowledge, this is the first demonstration that human UPF2 is essential for the NMD response in mammalian cells.

**DISCUSSION**

We have provided several lines of new evidence that the down-regulation of TCR-β transcripts in response to nonsense codons requires translation even though it occurs in the nuclear fraction of cells. We demonstrated that this down-regulation is mediated by an initiator ATG-dependent scanning process that requires the Kozak consensus sequence for optimal down-regulation (Figs. 1 and 3). This, along with our observation that the down-regulation can be triggered by an IRES (Fig. 2) and that it is reversed by specific suppressor tRNAs (19), strongly suggests that nonsense codons down-regulate TCR-β transcripts by a mechanism that depends on translation.

The simplest interpretation of our IRES data is that the NMD down-regulatory response requires a ribosome, although we cannot rule out that a non-ribosomal entity was recruited by the IRES in our experiments. To our knowledge, this is the first time that the down-regulatory response to a nonsense codon has been shown to be elicited by a 5' cap-independent mechanism. Several lines of evidence support the view that the IRES we used drives cap-independent translation and blocks cap-dependent translation. First, dicistronic mRNAs that contain the poliovirus IRES inserted between two reporter cistrons efficiently translate the second cistron without a requirement for ribosomes to traverse the first cistron (24). Second, introduction of small lesions throughout the central portion of the poliovirus IRES (region P) block cap-dependent initiation (35). Third, when the poliovirus IRES situated upstream of a reporter gene was debilitated by deletion or point mutation, translation of the reporter gene was extinguished, indicating that this IRES does not permit cap-dependent translation, probably because of its strong secondary structure (36). Fourth, translation of the reporter gene was extinguished, indicating that this IRES down-regulates transcripts in response to nonsense codons. To our knowledge, this is the first demonstration that human hUPF2 participates in the down-regulation of TCR-β transcripts in response to nonsense codons (Figs. 1 and 3). We therefore conclude that hUPF2 is an important component of the NMD RNA surveillance pathway in this down-regulatory response. However, we were not able to completely reverse TCR-β mRNA down-regulation, even when we cotransfected high concentrations of anti-hUPF2 plasmid (data not shown). This may reflect the limitations of antisense technology, but it could also indicate that TCR-β transcripts are down-regulated by two mechanisms, one that is hUPF2-dependent (the NMD RNA surveillance pathway) and another that is hUPF2-independent.

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