Translational Control of Tumor Necrosis Factor-related Apoptosis-inducing Ligand Death Receptor Expression in Melanoma Cells*

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In the present study, it was found that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-R2 protein expression did not correlate with mRNA expression in melanoma cell lines. In particular, early passage primary cultures from patients had low TRAIL-R2 protein expression compared with later passage cultures although TRAIL-R2 mRNA expression was similar in early and late passages. Similarly, cell lines made resistant to TRAIL by cultures in TRAIL had low TRAIL-R2 protein expression but normal levels of mRNA for TRAIL-R2. Expression from a luciferase reporter gene construct with the 3′-untranslated region (UTR) (but not the 5′-UTR) of TRAIL-R2 was suppressed when transfected into the TRAIL-selected (resistant) melanoma lines compared with that seen in the parental (sensitive) lines. Similar results were seen in early passage (resistant) cultures compared with late passage (sensitive) primary melanoma cultures. RNA gel shift assays demonstrated protein(s) binding to the 3′-UTR of TRAIL-R2 mRNA that were more evident in TRAIL-resistant cultures with low TRAIL-R2 protein expression. A 23-base fragment of the 3′-UTR inhibited binding of the proteins to the 3′-UTR, and a probe using this fragment bound to proteins in TRAIL-selected melanoma lines and early passage isolates of melanoma. Binding of the 3′-UTR probe to the cytosolic protein(s) was induced by exposure to TRAIL and was lost from the TRAIL selected lines 2–3 days after withdrawal of TRAIL from the cultures. These results are consistent with post-transcriptional regulation of TRAIL-R2 expression by cytosolic proteins induced by TRAIL that bind to the 3′-UTR region of TRAIL-R2 mRNA.

TRAIL\(^1\) is a member of the TNF family that, like TNF-\(\alpha\) and Fas ligand, is a type II membrane protein that can induce apoptotic cell death in a variety of cell types (1–3). TRAIL appears to be particularly important because it can induce apoptosis in a wide range of cultured malignant cells but not in normal tissues (4–9), with the possible exception of human liver cells (10). Normal human liver cells were reported to be sensitive to TRAIL, but this is believed to be due to the particular form of TRAIL used in those studies (10). Induction of apoptosis by TRAIL is believed to be mediated by interaction with two death receptors on cells referred to as TRAIL-R1 and TRAIL-R2 (see review in Ref. 3 for alternate nomenclature). Normal cells were postulated to be protected from TRAIL-induced apoptosis by their expression of TRAIL-R3 and TRAIL-R4, which lack cytoplasmic death domains and act to sequester TRAIL (decoy receptors) or to mediate antiapoptotic signals (6, 12).

We have shown previously that TRAIL was able to induce varying degrees of apoptosis in approximately two-thirds of the melanoma cell lines tested (4, 5). Sensitivity of melanoma cells to TRAIL-induced apoptosis showed an overall correlation with the level of death receptors, and in particular TRAIL-R2 expression, but did not correlate with the level of expression of the decoy receptors, TRAIL-R3 and TRAIL-R4 (5). Resistance of some cell lines to TRAIL was due to the absence of all receptors for TRAIL. Exposure of melanoma cells to TRAIL resulted in down-regulation of death receptors due to internalization in endosomes, and re-expression was from the trans-Golgi network. In contrast, the decoy receptors were located in the nucleus and underwent relocation to cytosol and cell membranes due to signals from the death receptors (13).

Factors that determine the level of TRAIL-R expression are not well understood. In some cell types, chemotherapy and irradiation was shown to up-regulate TRAIL-R2 expression by activation of p53 (14), whereas in others, p53-independent mechanisms were involved (15). Up-regulation of TRAIL-R2 by dexamethasone and interferon-gamma was independent of p53 (15). TRAIL-R1 (DR4) and TRAIL-R3 (DcR1) appeared to be also regulated by p53 (16, 17). The DNA binding sites for p53 were found to be located at three sites in the genomic locus of TRAIL-R2, either upstream of the ATG site or within intron 1 or intron 2 (18). The promoter region of the TRAIL-R2 gene was found to have transcription start sites 122 and 137 base pairs upstream of the initiation codon. Two Sp1 sites were responsible for the basal transcriptional activity (19), and it was speculated that agents binding to the Sp1 sites (such as certain histone deacetylase inhibitors) may up-regulate TRAIL-R2 expression. The promoter region for TRAIL-R1 (DR4) contained several AP-1 binding sites, which is a target for the c-Jun N-terminal kinase pathway that can be activated by several chemotherapeutic agents (20).

In addition to the variability of TRAIL-R2 expression between different melanoma lines, we reported that primary cultures established from patients commonly have low TRAIL death receptor expression but on successive culture passages acquire higher expression levels of TRAIL-R2 and sensitivity to TRAIL-induced apoptosis comparable with that seen in established lines (21). We also found that exposure to TRAIL over

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\(^1\) The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF, tumor necrosis factor; TRAIL-R, TRAIL receptor; PE, phycoerythrin; RACE, rapid amplification of cDNA ends; UTR, untranslated region; ARE, AU-rich element.
several weeks resulted in down-regulation of TRAIL receptors and that re-expression occurred over several weeks in the absence of TRAIL. These are referred to as TRAIL-selected resistant cells (21). The basis for down-regulation of TRAIL-R2 in the latter cell lines and in primary cultures is unknown, but we hypothesized that it may be due to transcriptional down-regulation of TRAIL-R2. To answer these questions, we examined TRAIL-R2 mRNA expression by real time PCR and correlated this with TRAIL-R2 protein expression. The results suggest that post-transcriptional events may be more important than transcriptional events in regulation of TRAIL-R2 expression.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Human melanoma cell lines Me4405, Mel-FH, Mel-RM, Mel-CV, MM200, Mel-1007, Mel-RMu, IgR3, Mel-LT, Mel-AT, Me10538, and SK-Mel-28 have been described previously (4, 5). The TRAIL-selected lines of Mel-FH, Mel-RM, and MM200 are described elsewhere (21). The cell lines were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia).

**Antibodies, Recombinant Proteins, and Other Reagents**—Recombinant human TRAIL (lot 6521-19) was supplied by Immunex (Seattle, WA). The preparation was supplied as a leucine zipper fusion protein, which required neither cross-linking nor proteolytic treatment in order to produce the mature TRAIL form. cDNA of TRAIL-1-R1 (IgG2a hu TR1-M271; lot 7136-07), TRAIL-R2 (IgG1 hu TRAIL-2-M413; lot 5274-96), TRAIL-R3 (IgG1 hu TR3-M430; lot 7313-217), and TRAIL-R4 (IgG1 hu TR4-M444; lot 7136-15) were supplied by Immunex and have been described previously (22). Flow Cytometry—Analysis was carried out using a Becton Dickinson (Mountain View, CA) FACSscan flow cytometer, as described previously (5). The percentage of antigen-positive cells was calculated as the difference in positive area between the positive and negative control histograms. The positive area was that to the right of the intersection of the two curves (23).

**Apoptosis**—Apoprotic cells were detected by the propidium iodide method described elsewhere (4, 5). In some experiments, apoptosis was measured by staining with PE-conjugated annexin V according to the manufacturer’s instructions. In brief, cells with or without pretreatment with TRAIL were washed twice with cold phosphate-buffered saline and then resuspended in binding buffer at a concentration of 1 × 10⁷ cells/ml. One hundred µl of the resulting solution (1 × 10⁷ cells) were transferred to a 5-ml culture tube, and 5 µl of annexin V-PE was added. After incubation at room temperature for 15 min in the dark, an additional 400 µl of binding buffer was added to each tube, and cells were analyzed by flow cytometry within 1 h.

**Real Time PCR**—Real time PCR was performed using the ABI Prism 7700 sequence detection system (PerkinElmer Life Sciences). A total volume of 25 µl was used, made up of 5 µl of the resulting solution (1 µl aliquots of the following: 0.2 mM of each primer, 0.2 mM of dNTP, 2.5 units of AmpliTaq Gold, 50 mM KCl, 7.5 mM MgCl₂, 0.01% BSA, and 1 µg of genomic DNA). The RNA is then treated with tobacco acid pyrophosphatase to remove the cap structure from full-length mRNA to produce a 5’-monophosphate end. A 45-base RNA adapter containing two forward primer sequences (see the protocol supplied by the manufacturer) is ligated to the RNA population using T4 RNA ligase. A random primer reverse transcriptase-PCR and a nested PCR then amplified the 5’-end of the specific RNA. Two reverse primers for the nested PCR are (5’-GAGAAGCACTCATCTACGCTCTC (outer reverse primer) and 5’-GGTGGATCAGCAGCACAGTCA (inner reverse primer)). For 3’ RACE, the first strand cDNA was synthesized from above total RNA using a supplied 3’-end adapter containing two reverse primer sequences (as per the protocol supplied by the manufacturer). Sequentially, nested PCR was performed on the resulting cDNA with other two primers, which were specific to the 3’-end region. The forward and reverse primers for the TRAIL-R2 5’-UTR. The inserts of 5’-UTR were produced by PCR with a primer pair of TGGGGCAAGACGTTGTTTCGCTTGTTGTATG (containing HindIII) and GTTGGTCATGGCCTGGAAGCGCTTATA (containing Ncol) and the above pGEM-T-3’-end as a template. The above insert was ligated into the linearized pGL3-promoter to form pGL3-5’-UTR.

Similarly, the 3’-UTR and 3’-UTR-invert insert were cloned by PCR with pGEM-T-3’-end as a template but with invert restriction sites. Then TRAIL-R2 3’-UTR and 3’-UTR-invert were ligated to the position of the deleted 3’-UTR and poly(A) (from 1934 to 2196) of the luciferase gene of pGL3-promoter by digestion with restriction enzymes of XbaI (for 3’-UTR) and, thus, pGL3-3’-UTR and pGL3-3’-UTR-invert were formed. Primers used were as follows: 3’-UTR forward primer (containing SpeI), GAAGACATCTGTTGAGGACACTTGGTGTAG; 3’-UTR reverse primer (containing BamHI), CCGGGATCCCCGCGAGCGAGAATTACGCA; 3’-UTR-invert forward primer (containing BamHI), GAAGCGGATCTCGAGGACACTTGGTGTAG; 3’-UTR-invert reverse primer (containing SpeI), TGGTATATCTGAGGACACTTGGTGTAG. The AU-rich element (ARE) insert was synthesized commercially (Sigma). Sense and antisense sequences were annealed by heating at 95 °C and cooled down to room temperature in annealing buffer (100 mM Tris-Cl, 100 mM NaCl) in 1 h. The double stranded DNA was ligated into the 3’-UTR and poly(A)-deleted pGL3-promoter as above. Ten micrograms of ARE (M-ARE) and M-ARE-deleted ARE (M-ARE-deleted ARE) fragments were created by the same methods except that Ts in the ARE region were changed into Cs. All of the above inserts in the pGL3-promoter were verified by sequencing with the above primers.

The above 3’-UTR and ARE inserts were also cloned into pGEM-T and pdcDNA3 as above for the purpose of synthesis of the mRNA probe and transfection to Mel-RM.

**Dual Luciferase Reporter Assay**—The firefly luciferase reporter constructs were co-transfected in melanoma cells with Renilla luciferase reporter pRL-SV40 vector. Two million melanoma cells were electroporated with 30 µg of each vector. Forty-eight h after transfection, the cells were analyzed by the Dual Luciferase Reporter Assay System (Promega, Madison, WI). The firefly luciferase activity was performed sequentially in a Lumat LB 9501 luminometer (Bertold, Germany) after adding their substrates. The relative light units from firefly vectors were normalized with those from the Renilla vector according to the manufacturer’s instructions. Note: Renilla luciferase activity and Renilla luciferase activity were performed sequentially in a Lumat LB 9501 luminometer (Bertold, Germany) after adding their substrates. The relative light units from firefly vectors were normalized with those from the Renilla vector according to the manufacturer’s instructions.

**Preparation of 32P-Labeled RNA Probes—Transcription in vitro** was performed in a reaction mix containing 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.5 mM each ATP, GTP, and CTP, 12 µCi UTP, 50 µCi of [α-32P]UTP (10 µCi/mM), 50 mM Tris-Cl, 10 mM MgCl₂, and 5 units of T7 RNA polymerase using Riboprobe In Vitro Transcription Systems (Promega, Annandale, Australia). An irrelevant Escherichia coli cRNA was used for a negative control, and unlabeled competitor cRNA synthesized under the same conditions was used as a specificity control.

**Post-transcriptional Regulation of TRAIL Death Receptors**

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Preparation of Cytoplasmic Extracts (S-100 Fraction)—Cytoplasmic extracts were prepared from Mel-FH, Mel-FH-select, Mel-RM, and Mel-RM-select cells using a protocol modified from Ref. 24. Briefly, the cells were collected by a scraper from three 175-cm² flasks, washed twice with phosphate-buffered saline at 4 °C, and resuspended in two packed volumes of buffer A (10 mM HEPES [pH 7.9], 15 mM MgCl₂, 10 mM KCl, 0.1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and a mixture of other proteinase inhibitors (Protease inhibitor mixture; Roche Applied Science)) at 4 °C, lysed on ice by 20 strokes in a Dounce homogenizer. The nuclei were removed by centrifugation at 14,000 × g for 5 min in a bench microcentrifuge. The crude cytoplasmic extracts were mixed with 0.11 volume of buffer B (0.3 mM HEPES [pH 7.9], 1.4 mM KCl, and 0.0 mM MgCl₂) at 4 °C and centrifuged for 60 min at 80,000 × g using a SW 50.1 rotor in a Beckman XL-90 ultracentrifuge. The high speed supernatants were dialyzed against buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The supernatant was recovered and stored at −80 °C after the concentration was measured by the Bradford assay method (Bio-Rad).

RNA Gel Retardation—This was performed according to a modification of the protocol of Walker et al. (25). Ten μg of the above S-100 protein extracts were incubated with the indicated complementary RNA probes in a buffer containing 10 mM HEPES (pH 7.2), 3 mM MgCl₂, 5% glycerol, 1 mM dithiothreitol, rRNA 0.5 mg/ml, tRNA 0.5 mg/ml, 100 mM KCl, and 1 μl of 32P-labeled cRNA probes (5 × 10¹⁰ cpm/0.6 mg) in a total volume of 20 μl. To determine RNA-binding specificity, 2 μl (0.2 μg) of unlabeled, nonspecific, 3'-UTR and ARE probes were incubated with cell extract before 32P-labeled probes were added for RNA gel retardation. The reaction was carried out at room temperature for 30 min. RNase T1 was added at 1 unit per action to remove all labeled RNA not protected by bound protein and incubated for 15 min. One μl of 20 mg/ml heparin was added and incubated for a further 10 min. The samples were then resolved on 4% polyacrylamide gel (60:1 acrylamide to bisacrylamide) using 0.5× TBE as a running buffer. Gels were dried and exposed to Kodak film at −80 °C.

RNA T1 mapping was performed as described by Leibold and Munro (26). Briefly after RNA gel was isolated by electroelution from the native gel in 45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH 8.3, at 150 V for 3 h. Then the current was reversed for the last 5 min of electrophoresis. The electroelution buffer was followed by extraction with phenol after the addition of tRNA (25 μg/ml) and precipitation with ethanol. The isolated RNAs were digested to completion with 10 units of RNase T1 (Ambion). For comparison, intact 3'-UTR and a small fraction of 3'-UTR were also digested with RNase T1. The samples were denatured at 80 °C for 5 min with 7 μl of RNA gel loading buffer II (Ambion) and electrophoresed at 20% acrylamide gel (25 μM uracil) using 0.5× TBE as a running buffer. Gels were dried and autoradiographed.

Actinomycin D Chase Study—The 3′-UTR-pcDNA3 and pcDNA3 were transfected to Mel-RM with LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions and selected by G418 (300 μg/ml). The transfected cells then were treated with actinomycin D (0.5 μg/ml) for 0, 1, 2, 3, and 2 h. The total RNA was extracted at different time points, and real time PCR was performed for TRAIL-R2 and glyceraldehyde-3-phosphate dehydrogenase mRNA.

RESULTS

TRAIL-R2 mRNA Expression in Relation to TRAIL-R2 Protein Expression and Sensitivity to TRAIL-induced Apoptosis—We examined the relation between TRAIL-R2 mRNA, TRAIL-R2 protein, and apoptosis in a panel of nine melanoma lines that exhibited a range of sensitivity to TRAIL-induced apoptosis. As reported previously (5), there was an overall correlation between TRAIL-R2 protein expression and sensitivity to TRAIL-induced apoptosis (Fig. 1a). There was, however, very little correlation between TRAIL-R2 protein expression and mRNA levels (e.g. in Me4405, there was high TRAIL-R2 protein and apoptosis but relatively low mRNA levels, whereas Mel007 had high TRAIL-R2 mRNA levels and relatively low TRAIL-R2 protein). The latter was also the pattern shown in studies on melanocytes.

Dissociation of Protein and mRNA Expression for TRAIL-R2 in Early Versus Late Passage Primary Melanoma Cultures from Patients—Fresh isolates of melanoma cells from patients frequently had relatively low TRAIL receptor expression, but expression of the latter increased with successive passages in culture (21). We examined mRNA levels for TRAIL-R2 in relation to TRAIL-R2 protein in successive passages from four patients. As shown in Fig. 1b, TRAIL-induced apoptosis and...
TRAIL-R2 protein was low or absent in passage 1 cultures and showed small to moderate increases with successive passages. mRNA values for TRAIL-R2 were, however, relatively constant throughout. In patient RW, mRNA levels were highest in passage 2 cells and then decreased with successive passages, whereas TRAIL-R2 protein was highest in passage 8 cells. In patient MC, TRAIL-R protein was only detected in the late passage 7 cells, whereas TRAIL-R2 mRNA was relatively constant in successive passages. In melanoma cultures from patient KC, mRNA for TRAIL-R2 showed an increase from passage 1 to passage 6 cells, but no significant TRAIL-R2 protein was detected until passage 6. There was no significant variation in the expression of HLA class I antigens detected by the monoclonal antibody W6/32 on melanoma. The percentage expression was 96.5% on RW passage 3 and 97.7% on RW passage 8.

Cultures from RW were sensitive to TRAIL-induced apoptosis. Those from MC, DC, and KC were relatively resistant. MC melanoma cells expressed relatively few receptors, but cultures from DC and KC were resistant even in late passage cultures expressing modest levels of TRAIL-R2 protein.

**TRAIL-R2 mRNA Levels in Melanoma Cells Selected for Resistance to TRAIL**—Culture of melanoma cells in TRAIL for prolonged periods results in generation of TRAIL-resistant cultures that have low TRAIL-R expression (21). Culture of the resistant lines in the absence of TRAIL resulted in partial recovery of both receptor expression and sensitivity to apoptosis. We examined the association between TRAIL-R2 protein and mRNA in these cells. The results in Fig. 1c indicate that mRNA from Mel-RM was at similar levels in the parental and resistant TRAIL-selected lines despite relatively low TRAIL-R2 protein. In the Mel-FH and MM200 lines, there was a reduction in both TRAIL-R2 mRNA and protein, but the reduction in TRAIL-R2 protein was relatively greater. There was no significant variation in the expression of the HLA class I antigen detected by monoclonal antibody W6/32 on Mel-FH, Mel-FH-select (99.1%, 99.4%) and Mel-RM, Mel-RM-select (98.5%, 98.5%).

**TRAIL-selected Resistant Melanoma Lines and Early Passage Melanoma Suppression Expression of Reporter Genes Containing 3'-UTR from the TRAIL-R2 mRNA**—The evidence presented above suggested that events controlling translation of protein from mRNA for TRAIL-R2 were an important determinant of TRAIL-R2 receptor expression. To investigate this further, we investigated expression from reporter constructs transfected into the cell lines. Reporter plasmids were constructed that used an SV40 early promoter-driven luciferase gene with the 3'-UTR from the TRAIL-R2 gene (shown in Fig. 2). The reporter constructs were transfected into the parental (Mel-FH, Mel-RM) and TRAIL-selected melanoma lines (Mel-FH-select and Mel-RM-select) and into early (P3) and late passage (P9) cultures from MC and RW (P2 and P8). As shown in Fig. 3a, firefly luciferase expression relative to *Renilla* luciferase expression was much lower in transfectants of early compared with late passage cultures from Mel-MC and Mel-RW (p = 0.005 for Mel-RW p8 versus p2; p = 0.006 for Mel-MC p9 versus p3). No significant differences in expression were seen in melanoma cells transfected with the vectors containing the 5'-UTR from TRAIL-R2 mRNA or the reverse sequence of the 3'-UTR (R2-invert). Similarly, when the two TRAIL-selected lines (Mel-FH-select and Mel-RM-select) and their parental lines were transfected with the pGL3-3'-UTR vector, luciferase expression was much less than the TRAIL-selected lines compared with expression from the parental lines (Fig. 3b). (By t test, p = 0.002 for Mel-FH versus Mel-FH-select, and p = 0.02 for Mel-RM versus Mel-RM-select.) No significant differences were seen in expression from lines transfected with the vector containing the 5'-UTR of TRAIL-R2 or the vector containing the reverse RNA sequence of the 3'-UTR (R2-invert).

**Proteins Binding to the 3'-UTR of TRAIL-R2 in RNA Gel Shift Assays**—The above results were considered consistent
with regulation of translation by binding of proteins to the 3'-UTR of the mRNA for TRAIL-R2. To examine this, RNA probes were synthesized by in vitro transcription and labeled with [α-32P]CTP. The probes were then mixed with cytosolic extracts of the melanoma cells and examined using RNA electrophoretic mobility shift assays.

Analysis of extracts from the Mel-RM and Mel-FH lines, together with their matching TRAIL-selected lines with low TRAIL-R2 protein expression, including Mel-FH-select, Mel-RM-select, Mel-MC passage 3, and melanocytes. Three RNA-protein complexes were detected. The upper band was not inhibitable by the unlabeled 3'-UTR probe and represents nonspecific binding to cytosolic proteins. The middle (row 2) complex was only detectable in the TRAIL-insensitive cells having low TRAIL-R2 protein expression, including Mel-FH-select, Mel-RM-select, Mel-MC passage 3, and melanocytes. The binding of protein(s) shown in row B3 is inhibitable by unlabeled 3'-UTR and exists in all of the cell lines.

Identification of proteins binding to the 3'-UTR of mRNA for TRAIL-R2. RNA gel retardation assay using [32P]-labeled 3'-UTR of R2 and cytosolic extracts from Mel-FH, Mel-FH-select, Mel-RM, Mel-RM-select, Mel-MC passage 3 and 9, RW passage 3 and 9, and cultured melanocytes. Three RNA-protein complexes were detected. The upper band was not inhibitable by the unlabeled 3'-UTR probe and represents nonspecific binding to cytosolic proteins. The middle (row 2) complex was only detectable in the TRAIL-insensitive cells having low TRAIL-R2 protein expression, including Mel-FH-select, Mel-RM-select, Mel-MC passage 3, and melanocytes. The binding of protein(s) shown in row B3 is inhibitable by unlabeled 3'-UTR and exists in all of the cell lines.

Protein Binding to ARE Is Related to Exposure of the TRAIL-selected Lines to TRAIL—We have shown previously that TRAIL-selected lines recover TRAIL-R2 expression several days after culture in the absence of TRAIL (21). We therefore examined whether this may be associated with loss of ARE-binding protein(s) after withdrawal of TRAIL. The study in Fig. 6, a and b, shows an increase in TRAIL-R2 protein expression commencing on day 1 and maximal by day 3 for both Mel-FH-select and Mel-RM-select lines. The ARE-binding activity was apparent up to day 1 for Mel-FH-select and day 2 for Mel-RM-select. (Faint binding was evident on day 2 and day 3 for Mel-FH-select and Mel-RM-select, respectively.) Conversely, when the TRAIL-selected lines were grown in the absence of TRAIL for 5 days and then re-exposed to TRAIL, there was a rapid reduction in TRAIL-R2 protein expression evident by 2 h (Fig. 6, c and d). Reappearance of protein binding to the labeled ARE was evident by 6 h. We have reported previously that TRAIL receptors undergo rapid endocytosis within 30 min of exposure to TRAIL (13), which may account for the apparent lag between reduction in TRAIL-R2 protein expression and the appearance of the protein binding to the ARE.

We examined whether expression of the ARE may change the stability of the mRNA for TRAIL-R2. To answer this, we made permanent transfectants with the vector containing the ARE in the Mel-RM and Mel-RM-selected melanoma cells and tested stability by measuring mRNA levels by real-time PCR after pulsing the melanoma cells with actinomycin D. As shown in Fig. 6, the half-life of the mRNA for TRAIL-R2 was increased in the cells transfected with the ARE compared with cells transfected with the mutant ARE or the vector alone. t for mRNA in Mel-RM was 1.5 h in the untreated cells, 1.49 h in the control vector-transfected cells, and 2.30 h in the ARE-transfected cells. Similarly, in the Mel-RM TRAIL-selected cells the t was 1.10 h for the untreated cells, 1.10 h in the control vector-transfected cells, and 2.15 h for the ARE-transfected cells. t for the control glyceraldehyde-3-phosphate dehydrogenase mRNA was 9.90 h in both the parental and TRAIL-selected cell lines.

**DISCUSSION**

We have reported previously that sensitivity of melanoma cells to TRAIL-induced apoptosis is determined predominantly by the level of TRAIL-R2 death receptor expression (5). TRAIL-R1 expression on melanoma cells may also mediate TRAIL-induced apoptosis but was not expressed as frequently as TRAIL-R2 (5). In the present study, we have therefore focused on TRAIL-R2 expression. We show that the level of TRAIL-R2 protein expression was not closely associated with the level of TRAIL-R2 mRNA expression. In particular, fresh isolates of melanoma cells and melanocytes with low levels of TRAIL-R2 protein expression and low sensitivity to TRAIL-induced apoptosis, had normal TRAIL-R2 mRNA levels. With
successive passages, the freshly isolated melanoma cells acquired TRAIL-R2 protein expression and increased their sensitivity to TRAIL. The present studies show that there was relatively little change in mRNA expression during culture, suggesting that post-transcriptional events were key determinants of TRAIL-R2 expression. Similar findings were made in studies on melanoma cells made resistant to TRAIL by prolonged culture in TRAIL. The latter had low TRAIL-R2 expres-
sion and sensitivity to TRAIL-induced apoptosis but normal levels of mRNA for TRAIL-R2. We reported previously that normal melanocytes appear to be protected from TRAIL-induced apoptosis because of low TRAIL death receptor expression (9). The existence of normal TRAIL-R2 mRNA levels but low TRAIL-R2 protein expression in melanocytes suggests that this may be a physiological control mechanism adapted by melanoma cells for their survival.

Regulation of translation has been shown to be important in many systems including production of ferritin (25) and up-regulation of p21 by epithelial growth factor (27, 28). In the case of TNF-α, it was shown that a protein of ~50,000 daltons bound to the 3'-UTR of the mRNA for TNF-α and regulated TNF-α protein production. The protein was increased in response to TNF-α and bound to AU-rich elements in the 3'-UTR (29).

In view of these studies, we examined whether similar events may be involved in regulation of TRAIL-R2 protein expression. Our results indicate that lysates of the TRAIL-resistant fresh isolates of melanoma and the TRAIL-selected melanoma lines had protein(s) that bound to 32P-labeled 3'-UTR from TRAIL-R2 mRNA in gel shift assays. These proteins
were not detectable in matching melanoma cells with normal TRAIL-R2 protein expression. Furthermore, it was shown that expression from luciferase reporter constructs containing the 3′-UTR of TRAIL-R2 mRNA was inhibited when transfected into the TRAIL-resistant cells with low TRAIL-R2 protein compared with expression from the reporter constructs transfected into melanoma cells with normal TRAIL-R2 protein expression. We identified the binding site of the protein in the 3′-UTR of TRAIL-R2 by RNase T1 mapping and showed that a 23-base fragment identified by this method inhibited binding of one of the proteins to the whole 3′-UTR probe. A probe made from this sequence bound to proteins in melanoma cell lysates in the same pattern as the whole 3′-UTR probe, and luciferase expression from reporter constructs containing this sequence was inhibited similar to that seen from constructs containing the whole 3′-UTR. These results support the view that TRAIL-R2 protein expression was regulated by protein(s) binding to a 23-base region from the 3′-UTR of the mRNA for TRAIL-R2. Negative regulatory effects may be mediated by the 5′-UTR (reviewed in Ref. 28), but we could find no evidence for involvement of this region in regulation of TRAIL-R2 expression.

The sequence identified in the 3′-UTR of TRAIL-R2 mRNA is rich in AU elements, and a number of proteins have been identified by others that might bind to this region. Multiple proteins were shown to bind to the 3′-UTR of p21. These included HuR, a member of the Drosophila ELAV family, and poly(C)-binding protein (CP1) (27). Both HuR and HuD are known to bind to AU-rich elements in the 3′-UTR and to stabilize mRNA (30). Studies on TNF-α production in macrophages found that two proteins (TIA-1 and TIAR) bind to the 3′-UTR of mRNA for TNF-α and silenced its translation. Another protein, tristetraplin, bound to the 3′-UTR of mRNA for TNF-α in activated macrophages and destabilized the mRNA (31). In the present studies, supershift assays with antibodies to HuR and HuD did not affect the migration of proteins binding to the ARE identified in the present study (data not shown). Actinomycin D chase studies on Mel-RM indicated that the half-life of mRNA for TRAIL-R2 was shorter in the TRAIL-selected lines than the parental or vector control lines and was increased in lines transfected with the ARE. These results are consistent with destabilization of the mRNA by the protein(s) binding to the ARE sequence in the 3′-UTR of TRAIL-R2.

Another question relates to the pathways that may be involved in regulation of the protein(s) binding to the 3′-UTR of TRAIL-R2. The substrate of p38 mitogen-activated protein kinase (32, 33). Whether similar pathways are involved in regulation of TRAIL-R2 protein expression is as yet unknown. We found that removal of TRAIL from cultures of TRAIL-selected melanoma cell lines was associated with loss of the 23-base ARE binding activity in cytosolic extracts from the cells with kinetics that approximated increased expression of TRAIL-R2 protein. Conversely, re-exposure of the TRAIL-selected lines to TRAIL resulted in reappearance of the proteins binding to the ARE. It is therefore possible that TRAIL itself may be one of the factors regulating the appearance of the ARE binding activity. Studies on this aspect are continuing.

The results would appear to have implications for therapeutic interventions that might increase translation of TRAIL-R2 protein (e.g. immunomodulatory peptides were reported to inhibit binding of proteins to the 3′-UTR mRNA for TNF-α) (34). Inhibitors of signal pathways involved in regulation of the protein binding to the 3′-UTR may also up-regulate the TRAIL-R2 protein. Whether such approaches might up-regulate receptors on normal tissue is as yet unknown.
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