Increased Expression of the E3-Ubiquitin Ligase Receptor Subunit βTRCP1 Relates to Constitutive Nuclear Factor-κB Activation and Chemoresistance in Pancreatic Carcinoma Cells

Susanne Müerköster, Alexander Arlt, Bence Sipos, Maike Witt, Maike Großmann, Günter Klöppel, Holger Kalthoff, Ulrich R. Fölsch, and Heiner Schäfer

1Laboratory of Molecular Gastroenterology and Hepatology, First Department of Medicine, Department of Pathology, and 2Molecular Oncology Research Group, Department of General Surgery, Kiel University, UKSH Campus-Kiel, Kiel, Germany

Abstract

The permanent activation of the transcription factor nuclear factor-κB (NF-κB) in pancreatic cancer cells is associated with a profound resistance towards chemotherapy. In the present study, we show that chemoresistant pancreatic cancer cell lines exhibiting constitutive NF-κB activity (i.e., PancTu-1, BxPc3, and Capan-1) express significantly elevated levels of the E3-ubiquitin ligase receptor subunit βTRCP1, compared with pancreatic carcinoma cell lines lacking constitutive NF-κB activity and chemoresistance (i.e., P45-T1 and T34M). In transfected with βTRCP1, PT45-T1 cells exhibit an elevated NF-κB activity and become less sensitive towards anticancer drug treatment (i.e., etoposide). Conversely, blockade of βTRCP1 expression in PancTu-1 cells by transfection with a vector-expressed small interfering RNA reduces NF-κB activation and chemoresistance. In PancTu-1 cells, βTRCP1 expression is inhibited, at least in part, by the interleukin-1 (IL-1) receptor(1) antagonist, whereas stimulation of PT45-T1 cells with IL-1β resulted in an increased expression of βTRCP1, and transfection of this cell line with βTRCP1 induced IL-1β secretion in a NF-κB-dependent fashion. Thus, via its close and mutual link to IL-1β secretion, βTRCP1 expression might substantially contribute to the persistent, IL-1β-dependent activation of NF-κB in pancreatic carcinoma cells. In support of this, βTRCP1 expression is detectable at considerable levels in a great number of pancreatic ductal adenocarcinoma specimens, along with an intense staining for activated NF-κB. Altogether, our findings of the elevated βTRCP1 expression in pancreatic carcinoma cells pinpoint to another important mediator of constitutive NF-κB activation and thereby of chemoresistance. (Cancer Res 2005; 65(4): 1316-24)

Introduction

A sustained elevation of nuclear factor-κB (NF-κB) activity is an abundant phenomenon of malignant tumors including leukemias and lymphomas as well as solid tumors, such as prostate, colorectal, and pancreatic carcinoma (1–4). As a major consequence of this constitutive activation of NF-κB, tumor cells express a great variety of NF-κB target genes conferring considerable growth advantages, such as antiapoptotic (i.e., cIAP and Bel-2) and cell cycle promoting (i.e., cyclinD) genes. In addition, permanent activation of NF-κB is associated with a profound resistance towards chemo- and radiotherapy (5, 6). In this way, pancreatic carcinoma represents a particularly threatening malignant disease inasmuch as pancreatic cancer cells are notoriously resistant to all known anticancer drugs such as gemcitabine, 5-fluorouracil, or etoposide (4, 7). However, from the close association of a chemoresistant phenotype and an elevated NF-κB activity, new concepts of chemosensitization have been proposed that rely on specific inhibition of NF-κB activation (5, 6). These concepts include substances such as proteasome inhibitors (i.e., MG132 and YPD431) or IκB kinase inhibitors (i.e., sulfasalazine; refs. 5–9). To confine the suitability of NF-κB as a new molecular target in anticancer therapy, a better understanding of the molecular mechanisms leading to constitutive NF-κB activation is still a major goal. Besides certain genetic alterations such as IκB gene mutations and Rel-gene amplifications (1–12), many signaling pathways upstream of the IκB kinase have been supposed to amplify NF-κB activation, including rasraf1 (13), mitogen-activated protein kinase (14–16), or Akt/PKB (17).

However, in pancreatic carcinoma cells other mechanisms seem also to be involved (18). From recent studies, evidence accumulated that the autocrine and juxtacrine actions of certain cytokines, such as interleukin-1β (IL-1β), confer permanent NF-κB activation in pancreatic carcinoma along with a profound chemoresistance (19, 20). Furthermore, other molecular and cellular conditions are conceivable as a prerequisite for the maintenance of high NF-κB activation. This particularly includes molecules involved in the second step of NF-κB activation—the degradation of IκBα. As a consequence of the signal induced IκB kinase-mediated phosphorylation of IκBα [i.e., in response to tumor necrosis factor-α (TNFα) or IL-1β], the inhibitory protein of NF-κB becomes rapidly polyubiquitinated and then degraded by the 26S proteasome (21). The substrate-specific polyubiquitination of phospho-IκBα represents the rate-limiting step in the signal-driven turnover of IκBα and is executed by the E3-ubiquitin ligase multi-protein complex, also known as Skp1-Cullin-F-box protein complex (22). The substrate specificity of IκBα polyubiquitination depends on the 67 kDa F-box protein βTRCP1 (β-transducin repeat–containing protein) and its closely related homologue βTRCP2/homologue of Slimb1 (βTRCP2/HOS; ref. 23). These two proteins bring phospho-IκBα into the right position for polyubiquitination via the RING E3-ligase Rbx. Whereas HOS is particularly expressed in the cytoplasm (24), βTRCP1 is predominantly expressed in the nucleus (24, 25) where it is part of the recently described intranuclear regulation of the IκBα shuttling (reviewed in ref. 26). An elevated expression of these F-box proteins may cause a permanent derepression of NF-κB activation, as reported for pancreatic cancer cells (7).
We herein investigated the role of βTRCP1 in the constitutive NF-κB activation in pancreatic cancer cell lines and the development of chemoresistance in these cells. Our findings indicate an important contribution of βTRCP1 expression to the NF-κB-dependent chemoresistance, and therefore allow a better understanding of this particular phenotype.

Materials and Methods

Cell Lines and Culture. The human pancreatic carcinoma cell lines Panc-Tu1, BxPc3, Capan-1, T3M4, and PT45-P1 and their handling were described previously (7, 27, 28). Cells were kept in culture (37°C, 5% CO2, 85% humidity) using RPMI-40 (PAΑ Laboratories, Colbe, Germany) supplemented with 1% glutamine (Life Technologies, Eggenstein, Germany) and 10% FCS (Biochrom KG, Berlin, Germany).

Reagents. Etoposide was from Bristol-Myers-Squibb (Munich, Germany). TNFα, bovine serum albumin, sulfassalazine, and Leptomycin B were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Recombinant human IL-1β and human IL-1 receptor(I) antagonist were obtained from R&D Systems (Wiesbaden, Germany). MG132 was from Biomol (Hamburg, Germany) and Annexin V/propidium iodide (PI) was from Biocarta (Hamburg, Germany).

Generation of βTRCP1 siRNA and βTRCP1 Expression Vectors. Synthetic ssDNA oligonucleotides encoding duplexed siRNA targeting to βTRCP1 mRNA (position 441-461: 5'-tctggtagttacacatgttggaccc-gctggttgattgaacacttt-3'/5'-ctataaagggctcagtagttttggtcg-catgtggttgaacc-3'), or encoding a scrambled control RNA: 5'- tctggtagttacacatgttggaccc-gctggttgattgaacacttt-3'/5'-ctataaagggctcagtagttttggtcg-catgtggttgaacc-3', were both flanked by restriction sites for 5'Sal/3' XbaI were annealed and then ligated into the pSHH1 expression vector (Imgenex). The coding region of βTRCP1 mRNA (Genbank accession no. Y14153) from PancTu-1 cells was cloned via reverse transcription-PCR (RT-PCR; forward/reverse primers: 5'-aggctccgaggtat-tggac-5', position 54/5'-gctcgtgattgatattggc-3', position 1,796) into the pCR3.1 expression vector (Invitrogen, Karlsruhe, Germany). All constructs were checked by automated DNA-sequencing (ABI Prism 3770).

Cell Transfection. PT45-P1 and PancTu-1 cells, seeded into six-well plates (2.5 × 105 cells/well), were grown overnight, followed by mono- or cotransfection with 5 μg/well of DIMRIE reagent (Invitrogen) and 1 μg/well of the following plasmids: pcDNA3.1-βTRCP1, pcDNA3.1-ΔNk-Bx (h-Bu superrepressor; ref. 7), pcDNA3.1-lacZ, pSHH1-βTRCP1-siRNA, pSHH1-control-siRNA, and pSHH1-empty. Upon transfection for 8 to 16 hours, 1 mL medium containing 20% FCS was added and cells were left untreated for at least 8 hours before their further handling.

Preparation of Nuclear and Cytoplasmic Extracts. Hypotonic lysis of cells and subsequent fractionation into cytoplasmic and nuclear extracts were done as described (29, 30). The protein content of each fraction was determined by the D2-Protein assay (Bio-Rad Laboratories, Munich, Germany), and the quality of the separation in nuclear and cytoplasmic protein was checked by a commercial enzyme assay (Sigma-Aldrich) for lactate dehydrogenase (LDH) activity following the instructions of the manufacturer. LDH activity was almost completely retained in the cytoplasmic fraction (20-30 millunits/μg protein), whereas no significant LDH contamination was found in the nuclear material (<1 millunit/μg protein). For comparative Western blot analysis of nuclear and cytoplasmic extracts, stimulatory protein 1 and LDH, respectively, were used as protein loading control.

Gel-Shift and Supershift Assay. Nuclear extracts were incubated with the γP2-labeled oligonucleotide 5'-AGTGGGAGGTCCCAGCCG-3' containing a consensus NF-κB binding site (Promega, Mannheim, Germany). After incubation at room temperature (30 minutes), samples were separated by gel electrophoresis at 100 V, 4°C. Gels were dried and exposed to X-ray Hyperfilm (Amersham, Freiburg, Germany). For supershift assays, p65/p50 antibodies (Santa Cruz, Heidelberg, Germany) were added (1 hour, 4°C) before electrophoresis.

ELISA-Based Nuclear Factor-κB Assay. Additionally to gel-shift assays, an ELISA-based kit was used for quantitative detection of NF-κB activity (TransAM NF-κB kit, Active Motif Europe, Rixensart, Belgium). For each sample, 20 μL of nuclear extracts (= 5 μg protein) were used according to the instructions of the manufacturer. For the detection of activated NF-κB, antibodies against the p65/RelA or p50 subunit (Santa Cruz) were used, followed by a secondary antibody conjugated to horseradish peroxidase. The colorimetric readout (450 nm) was done with an ELISA plate-reader.

Interleukin-1β ELISA. To measure human IL-1β secretion, cell culture supernatants were precleared by centrifugation (10,000 rpm, 10 minutes) and submitted to the Quantikine-HS human IL-1β immunoassay (R&D Systems) following the instructions of the manufacturer. IL-1β concentrations were normalized to the cell numbers determined in parallel.

Measurement of Apoptosis. Cells (2.5 × 105) were stained with Annexin V/PI following the instructions of the manufacturer and analyzed by fluorescence flow cytometry (Galaxy/Argon Plus; DakoCytomation, Hamburg, Germany) using the FLOWMAX software. Cells exhibiting high specific Annexin V staining were regarded as apoptotic.

Immunoprecipitation and Western Blotting. Mock- or βTRCP1-transfected PT45-P1 cells (2 × 105) were resuspended in 500 μL of 50 mMol/L Tris-HCl (pH 7.4), 1% (v/v) Triton X-100, 150 mMol/L NaCl, 1 mL EDTA, 0.1 mMol/L phenylmethylsulfonyl fluoride (TNET buffer), 0.1 mMol/L Na3VO4, supplemented with protease inhibitor cocktail (Complete-Mini/EDTA-free, Roche, Mannheim, Germany), and lysed by sonification (3 × 10 seconds). Then, lysates were cleared by centrifugation (microfuge, 13,000 rpm for 10 minutes, 4°C) and adjusted to equal protein concentrations using the D2-Protein assay (Bio-Rad Laboratories). Afterwards, lysates were precleared by protein A-agarose beads at room temperature for 1 hour and the resulting supernatant was incubated overnight at 4°C with anti-IκB-agarose conjugate (C21, Santa Cruz). The beads were recovered by centrifugation, washed five times with TNET buffer, and prepared for electrophoresis in 50 μL SDS sample buffer (95°C, 5 minutes). For Western blotting, nuclear extracts or total cell lysates in SDS sample buffer (without bromphenol blue) were prepared and protein concentrations were determined. Ten micrograms of protein were used of each sample and an appropriate volume of SDS sample buffer containing 0.2 mg/mL bromphenol blue and 2.5% β-mercaptoethanol was added. These samples or the immunoprecipitated proteins were run on SDS-PAA gels (+20% gradient) and electroblotted as described previously (18). For the detection of βTRCP1 and βTRCP2/HOS, a polyclonal goat antibody (N15 and C20, Santa Cruz) was used at a concentration of 1 μg/mL in 0.05% Tween 20 in TBS (TBST) containing 2% nonfat milk powder (2% Skim-TBST). Polyclonal antibodies were also used for the detection of phospho-IκBα (Cell Signalling, Frankfurt, Germany) and IκBα (C21, Santa Cruz) at 0.1 and 0.4 μg/mL in 5% bovine serum albumin-TBST, respectively, and polyubiquitinated IκBα was detected with a monoclonal polyubiquitin antibody (clone P4D1, Santa Cruz) at 0.2 μg/mL in 2% skim-TBST. As control of equal protein load, an α-tubulin monoclonal antibody or a LDH monoclonal antibody (Sigma-Aldrich) was used at 0.01 μg/mL or a stimulating protein 1 antibody (Santa Cruz) at 0.2 μg/mL in 5% bovine serum albumin-TBST. All antibodies were incubated overnight at 4°C. For the detection of these primary antibodies, anti-goat (Santa Cruz), anti-mouse, and anti-rabbit horseradish peroxidase-linked antibodies (Cell Signaling) were used at a dilution of 1:2,000 in 5% skim-TBST at room temperature for 1 hour.

Reverse Transcription-PCR. Total RNA was isolated and reverse transcribed into single-stranded cDNA as described (31). PCR for the detection of βTRCP1 was conducted as follows: 2 μL of cDNA were submitted to thermal cycling (95°C, 2 minutes/95°C, 60 seconds; 58°C, 30 seconds; 72°C 30 seconds; for 25 cycles/72°C, 10 minutes) using 1.5 units of Taq polymerase (Invitrogen) and 0.5 μmol/L forward/reverse primers (5'-aggctccgaggtat-tggac-3', position 54/5'-gctcgtgattgatattggc-3', position 1,796). For control, β-actin was analyzed with RT-PCR amplifier-primer sets (BD Clontech, Heidelberg, Germany). PCR products were separated by 8% PAGE and visualized by ethidium bromide.

Immunohistochemistry. For βTRCP1 and NF-κB immunostaining, 26 ductal pancreatic carcinoma and five normal pancreatic tissue samples obtained from surgical specimens were used, according to a protocol approved by the ethics committee of the University Hospitals, Kiel (Permission no. 110/99). Samples were snap frozen in a mixture of
Avidin/Biotin-Blocking Kit (Vector Laboratories/Alexis, Grünberg, Germany). Serum blocking and detection were done with a peroxidase-based rabbit Vectastain Kit (Vector Laboratories). Rabbit polyclonal anti-βTRCP1 antibody (C18, Santa Cruz) was applied in 3 μg/mL concentration for 1 hour at room temperature. For negative control, anti βTRCP1 antibody was coincubated with 5-fold weight excess of specific blocking peptide (Santa Cruz) for 1 hour before immunostaining. A mouse p65/RelA antibody (Chemicon, Hofheim, Germany), recognizing the unmasked, activated NF-κB-subunit p65, was applied in 25 μg/mL concentration for 1 hour at room temperature and developed with mouse Envision Kit (DakoCytomation). The primary antibody was omitted for negative control. After staining, sections were counterstained in 50% haemalaun (Merck, Darmstadt, Germany) and mounted with glycerol gelatin. Immunohistochemical stains were evaluated by a semiquantitative method. βTRCP1 and NF-κB expression in tumor cells was rated as mild (<10% of cells stained), moderate (10-50%), or strong (>50%).

Results

Elevated βTRCP1 Expression Levels in Pancreatic Carcinoma Cell Lines Exhibiting Constitutive Nuclear Factor-κB Activation. By means of Western blotting, βTRCP1 expression was analyzed in various pancreatic carcinoma cell lines. Nuclear extracts from chemoresistant Capan-1, BxPc3, and PancTu-1 cells (7) exhibiting high constitutive NF-κB activity (Fig. 1A) were compared with those from chemosensitive PT45-P1 and T3M4 cells lacking constitutive NF-κB activity (Fig. 1B). As shown in Fig. 1B, a strong signal for βTRCP1 expression, corresponding to a 67 kDa protein, was detectable in Capan-1, BxPc3, and PancTu-1 cells. The specificity of this band was verified by antibody incubation in the presence of an excess of a blocking peptide (data not shown) as well as by transfection of βTRCP1 yielding a protein of identical

![Figure 1](image1.png)

Figure 1. Expression levels of βTRCP1 in pancreatic carcinoma cell lines exhibiting distinct basal NF-κB activities. Nuclear extracts of the human pancreatic carcinoma cell lines Capan-1, BxPc3, PancTu-1, PT45-P1 and T3M4 were submitted to (A) gel shift assay using a labelled specific consensus oligonucleotide for NF-κB binding, or (B) Western blotting for the detection of βTRCP1 (antibody N-15, Santa Cruz). The detection of α-tubulin was used as a control for equal protein load. Representative results from three independent experiments are shown.

![Figure 2](image2.png)

Figure 2. Effect of increased βTRCP1 expression on NF-κB activity and chemoresistance in PT45-P1 cells. PT45-P1 cells [untreated (ut)] were transfected with the empty vector (mock) or with βTRCP1. Nuclear extracts from these cells were submitted to (A) Western blotting for the detection of βTRCP1 or (B) NF-κB gel-shift/supershift assay. Representative results from three independent experiments are shown. C and D, PT45-P1 cells were mock-transfected or transfected with βTRCP1, either alone or in combination with pCMV-lacZ (as control) or pcDNA3.1 ΔN-h-Bad (Δ.BSR). C, mock- or βTRCP1-transfected PT45-P1 cells were either left untreated (w/o) or treated with 0.5 mmol/L sulfasalazine (24 hours). Nuclear extracts from these cells and from h-BSR/lacZ cotransfectants were analyzed by ELISA-based NF-κB binding assay [columns, mean (n = 4); bars, SD]. D, mock- or βTRCP1-transfected PT45-P1 cells were treated with etoposide (24 hours) or without, either alone or after preincubation (−1 hour) with 0.5 mmol/L sulfasalazine. PT45-P1 cells cotransfected with βTRCP1/mock together with h-BSR/lacZ were treated without or with 20 μg/mL etoposide for 24 hours. Apoptosis was determined by Annexin V/PI staining and subsequent flow cytometry. Data are presented as etoposide-induced apoptosis (%) over basal (columns, mean (n = 4); bars, SD).
size (see below). In striking contrast, βTRCP1 expression was not detectable at significant levels in PT45-P1 and T3M4 cells (Fig. 1B).

Effect of Increased βTRCP1 Expression on Nuclear Factor-κB Activity and Chemoresistance in PT45-P1 Cells. PT45-P1 cells lacking significant βTRCP1 expression were transfected with an expression vector for βTRCP1 or an empty vector (mock). As shown by Western blot (Fig. 2A), a 67 kDa protein was detected in nuclear extracts from βTRCP1 transfectants, similar in size to the protein identified in the resistant cell lines described above. To check whether βTRCP1 expression in PT45-P1 cells confers increased NF-κB activity, gel-shift assays were done. As shown in Fig. 2B, βTRCP1-transfected PT45-P1 cells exhibited a significantly stronger NF-κB binding activity than mock-transfected or untransfected PT45-P1 cells. Similarly, an ELISA-based NF-κB binding assay (TransAM, ActiveMotif), specifically detecting DNA-bound p65/p50, revealed an increased level of activated NF-κB in βTRCP1-transfected cells compared with mock transfectants (Fig. 2C), supporting the data revealed by gel-shift assay.

To elucidate whether overexpression of βTRCP1 influences the sensitivity towards anticancer drugs, PT45-P1 cells were transfected with βTRCP1 and apoptosis was determined by Annexin V/PI staining and subsequent fluorescence flow cytometry. As shown in Fig. 2D, mock-transfected PT45-P1 cells exhibited strong apoptosis (up to 50% apoptotic cells) on treatment with the anticancer drug etoposide (20 g/mL, 24 hours). In contrast, PT45-P1 cells transfected with βTRCP1 became much less sensitive towards anticancer drug-induced apoptosis (20-25% apoptotic cells). To verify that this desensitization by βTRCP1 is NF-κB-dependent, PT45-P1 cells were either cotransfected with the IκBα superrepressor or were pretreated with the IκBα kinase inhibitor sulfasalazine before etoposide incubation. As shown in Fig. 2D, the decreased sensitivity of βTRCP1 transfectants in response to etoposide treatment (20-25% apoptotic cells) was almost completely reversed by sulfasalazine (>45% apoptotic cells) and strongly inhibited by the IκBα superrepressor (35-40% apoptotic cells). In parallel, significant suppression of the elevated NF-κB activity in βTRCP1-transfected PT45-P1 cells by sulfasalazine or the IκBα superrepressor could be verified by the ELISA-based NF-κB binding assay (Fig. 2C).

Enhanced Turnover of IκBα in PT45-P1 Cells Transfected with βTRCP1. To elucidate whether the expression of βTRCP1 affects IκBα expression, transfected PT45-P1 cells were treated with TNFα and MG132, or not. Western blot analysis of total cell extracts from these cells (Fig. 3A) revealed significantly lower levels of IκBα in PT45-P1 cells transfected with βTRCP1 than in mock transfectants. This effect was already seen in untreated cells and was much more pronounced in cells treated with the proteasome inhibitor MG132 alone (45 minutes) or with TNFα alone (15 minutes), or in cells preincubated with MG132 (30 minutes) and treated with TNFα (15 minutes). The corresponding levels of phospho-IκBα were similarly reduced in βTRCP1 transfectants. Whereas in untreated cells no significant amount of phospho-IκBα could be detected, a fairly detectable phospho-IκBα band was present in MG132-treated cells, and this band was significantly weaker in βTRCP1-transfected PT45-P1 cells than in mock transfectants. The amount of phospho-IκBα in PT45-P1 cells treated with TNFα alone or with the combination of MG132 and TNFα was also lower if βTRCP1 was overexpressed. A similar βTRCP1-dependent pattern of the IκBα turnover has been recently found in 293T cells (22) or in murine cells (32).

Next, total cellular lysates from transfected PT45-P1 cells were submitted to immunoprecipitation using an anti-IκBα conjugate. Again, distinct levels of phospho-IκBα and IκBα were detectable in the immunoprecipitated material (Fig. 3B). In βTRCP1 transfectants, the level of phospho-IκBα already detectable in the presence of MG132 alone—indicating intrinsic IκBα-phosphorylation in unstimulated PT45-P1 cells—was significantly lower than in mock transfectants. In the presence of both MG132 and TNFα, strongly suppressed levels of phospho-IκBα were observed in PT45-P1 cells expressing βTRCP1 in comparison with mock-transfected cells. To further elucidate the influence of βTRCP1 transfection on IκBα polyubiquitination, the immunoprecipitated protein from

**Figure 3.** Enhanced turnover of IκBα and phospho-IκBα in PT45-P1 cells expressing βTRCP1. PT45-P1 cells were mock- or βTRCP1-transfected, followed by incubation with TNFα (30 ng/mL, 15 minutes), with MG132 (5 μmol/L, 45 minutes), or with TNFα upon preincubation (30 minutes) with MG132. A, total cell lysates were submitted to Western blotting using IκBα, phospho-IκBα, and tubulin antibodies. B, total cell lysates were immunoprecipitated with IκBα antibodies, submitted to Western blotting, and probed with IκBα or polyubiquitin antibodies; Ig-hc, immunoglobulin heavy-chain. C, mock- or βTRCP1-transfected PT45-P1 cells were incubated with Leptomycin B (LmB; 50 ng/mL, 16 hours) or not. Cytosolic and nuclear extracts were submitted to Western blotting for the detection of IκBα. The detection of LDH and stimulatory protein 1 was used as control for the purity of cytoplasmic and nuclear extracts and for equal protein load. The results shown are representative for two independent experiments.
MG132-treated cells (either alone or together with TNFα) was probed with a polyubiquitin-specific antibody. As shown in Fig. 3B, the amount of polyubiquitinated protein exhibiting high molecular weight (>100 kDa) was rather low in mock-transfected PT45-P1 cells treated with MG132 alone, whereas under these conditions a strong ubiquitin positive protein signal was detectable in βTRCP1 transfectants. This higher level of polyubiquitinated high molecular weight protein was also seen in βTRCP1-transfected PT45-P1 cells subject to MG132/TNFα coincubation, producing a much stronger polyubiquitination pattern in both transfectants.

To further investigate whether the reduced level of IκBα in βTRCP1-expressing PT45-P1 cells is the result of an enhanced nuclear turnover, mock or βTRCP1 transfectants were treated with Leptomycin B, an inhibitor of the nuclear exporter Crm-1, and the distribution of IκBα in nuclear and cytoplasmic fractions was analyzed. The purity of these fractions was confirmed by excluding significant LDH contamination in nuclear extracts and stimulatory protein 1 contaminations in cytoplasmic samples (30, 33). As shown by Western blotting (Fig. 3C), after Leptomycin B treatment an increase in the amount of IκBα in nuclear extracts from both transfectants occurred and, in turn, a decrease in cytosolic extracts. Of note, in βTRCP1-transfected PT45-P1 cells, the amount of nuclear IκBα was significantly lower than in mock-transfected cells, whereas this decrease in the amount of IκBα was not seen in cytosolic extracts upon Leptomycin B treatment.

Effect of Disrupted βTRCP1 Expression on Nuclear Factor-κB Activity and Chemoresistance in PancTu-1 Cells. Next, it was elucidated whether disruption of βTRCP1 expression in PancTu-1 cells affects the elevated basal NF-κB activity and the chemoresistance of this cell line. For this purpose, an expression vector for a βTRCP1-siRNA was generated and transfected into PancTu-1 cells. Western blot analysis (Fig. 4A) revealed that in total cellular extracts from these cells, the amount of the 67 kDa βTRCP1 protein was strongly reduced in comparison with PancTu-1 cells expressing a control-siRNA or with mock-transfected cells. In contrast, the smaller βTRCP-variant βTRCP2/HOS (58 kDa) was not affected by the βTRCP1-siRNA, thus underscoring its target specificity. The effect of disrupted βTRCP1 on NF-κB activity in PancTu-1 cells was further checked by gel-shift assay. As shown in Fig. 4B, the intensity of the detectable NF-κB-DNA complex was significantly decreased in PancTu-1 cells transfected with βTRCP1-siRNA compared with mock-transfected PancTu-1 cells or with cells transfected with the control-siRNA. This decreased NF-κB binding activity in βTRCP1-siRNA-transfected PancTu-1 cells was also verified by the ELISA-based NF-κB binding assay (Fig. 4C). As an obvious consequence of the disrupted βTRCP1 expression in PancTu-1 cells, the chemoresistance of this cell line was significantly decreased. As shown in Fig. 4D, PancTu-1 cells expressing the βTRCP1-siRNA exhibited a significantly higher rate of apoptosis in response to etoposide treatment (30% apoptotic cells) than transfectants.
Interrelationship of IL-1β secretion and βTRCP1 expression in PancTu-1 and PT45-P1 Cells. Owing to the elevated NF-κB activity in some pancreatic carcinoma cells recently shown to be related to the autocrine action of IL-1β, we elucidated the effect of IL-1β on βTRCP1 expression. As shown by Western blot (Fig. 5A, left) and RT-PCR analysis (Fig. 5B, left), treatment of PancTu-1 cells with the IL-1 receptor(I) antagonist (18 hours) or not, and nuclear extracts were analyzed by the ELISA-based NF-κB assay. E, mock- or βTRCP1-transfected PancTu-1 cells were treated with etoposide or without followed by administration of the IL-1 receptor(I) antagonist 8 hours thereafter, or not (w/o). Apoptosis was determined 24 hours after etoposide administration by Annexin V/PI staining and subsequent flow cytometry (C, E, columns, mean (n = 4); bars, SD).

βTRCP1 and NF-κB-Dependent Chemoresistance

TRCP1 expression is abundantly present in human pancreatic ductal adenocarcinoma specimens. To further investigate whether pancreatic carcinoma is characterized by an increased βTRCP1 expression, tissue sections of snap frozen surgical material from pancreatic cancer patients were analyzed by immunohistochemistry. In 17 of 26 analyzed specimens, pancreatic ductal adenocarcinomas exhibited significant βTRCP1 expression, as shown by the intensive and epitope-specific immunostaining with the βTRCP1 antibody (Fig. 6A). In 11 of these positively tested carcinomas, βTRCP1 expression was more abundant, as indicated by the dense immunostaining of 10% to 50% or even >50% of the tumor cells. In six tumor specimens, the density of βTRCP1 immunostaining was lower, as indicated by the detection of <10% positive tumor cells, and in nine tumor specimens no significant βTRCP1 expression was detectable. In comparison, all carcinoma specimens were positively tested for activated NF-κB (Fig. 6C), and more than 90% of these carcinomas were strongly stained (>50% of the tumor cells) with the antibody recognizing the unmasked, h-B-freed, p65/RelA subunit of activated NF-κB. In normal pancreatic tissue, no expression of βTRCP1 was found at all and activated NF-κB was detected in several cases in pancreatic acini (data not shown).
Discussion

Pancreatic ductal adenocarcinoma is characterized by an extremely malignant phenotype and a desperately poor prognosis. Mostly (>80%), the disease is diagnosed in an advanced stage and the only therapeutic options then rely on palliative chemotherapy treatments (34, 35). The major limitation in the efficacy of chemotherapy is the innate and acquired resistance of pancreatic carcinoma cells to apoptosis-inducing anticancer drugs. In this context, constitutive activation of NF-κB is of particular importance for a chemoresistant phenotype, as shown for the pancreatic ductal carcinoma cell lines PancTu-1, BxPc3, and Capan-1 (7, 18).

Our present study addressed the mechanisms of this constitutive NF-κB activation. We were able to show that chemoresistant pancreatic carcinoma cell lines are characterized by a significantly elevated expression of the E3-ubiquitin ligase receptor subunit βTRCP1 (Fig. 1). This protein is of particular importance for the activation of NF-κB as it recruits phosphorylated IκBα to the ubiquitin conjugating E3-ligase (SCF) complex, followed by the polyubiquitination of IκBα and its rapid degradation by the 26S proteasome (21, 22). As shown for PancTu-1 cells, down-regulation of βTRCP1 expression by siRNA caused a significant decrease in NF-κB activity and an increased sensitivity towards anticancer drugs such as etoposide (Fig. 4). Conversely, in those pancreatic carcinoma cell lines lacking high βTRCP1 expression, constitutive NF-κB activation, and chemoresistance (i.e., PT45-P1 cells), transfection with βTRCP1 strongly increased the NF-κB activity and conferred a more chemoresistant phenotype (Fig. 2). Preceding this altered phenotype, the turnover of IκBα along with its polyubiquitination was enhanced in βTRCP1-transfected PT45-P1 cells (Fig. 3), indicating a reduced NF-κB repression. Consequently, inhibition of these molecular events (i.e., by sulfasalazine or the IκBα superrepressor) abrogated the NF-κB-dependent chemoresistance induced by βTRCP1 (Fig. 2).

βTRCP1 is supposed to be involved in the intranuclear derepression of NF-κB activity (24, 25) that mainly affects the shuttling of free IκBα between the cytoplasm, where it prevents NF-κB from nuclear entry, and the nucleus where it retrieves nuclear NF-κB back to the cytoplasm. However, evidence accumulated indicating that even IκBα-bound NF-κB enters the nucleus, either via a nuclear localization signal of IκBα or an incompletely masked p50 subunit (26, 36, 37). However, this NF-κB/IκBα complex is unable to stay in the nucleus, due to being retrieved from there by the nuclear export sequence of IκBα (26, 38), unless intranuclear IκBα is recruited by βTRCP1 to the resident nuclear polyubiquitination complex, followed by its proteasomal degradation in the nucleus (24, 33). Another F-box protein, designated HOS (23), is predominantly expressed in the cytoplasm and is regarded as mediator of cytoplasmic polyubiquitination of IκBα and IκBβ. In those pancreatic carcinoma cell lines investigated in the present study, HOS (βTRCP2) expression was less different between chemoresistant and chemosensitive cell lines than βTRCP1 expression (data not shown), suggesting that the constitutive NF-κB activation seen in chemoresistant cells is rather due to the forced derepression of NF-κB activity in the nucleus by βTRCP1. This is supported by the finding that the enhanced IκBα turnover was more prominent in the nucleus of βTRCP1-transfected PT45-P1 cells subject to Leptomycin B treatment (Fig. 3C). Such an intranuclear derepression requires a preceding signal that induces IκBα phosphorylation. We have recently shown that PancTu-1 cells as well as several other pancreatic carcinoma cell lines sharing the chemoresistant and NF-κB positive phenotype possess an autocrine activation loop involving IL-1β (19, 20). It can be assumed that

Figure 6. Immunohistochemical stain of βTRCP1 and NF-κB in human pancreatic ductal adenocarcinomas. A representative immunohistochemical staining shows (A) strong βTRCP1 immunoreactivity in tumor cells; (B) negative control by preabsorption of the βTRCP1 antibody with a specific blocking peptide before staining; (C) strong immunoreactivity for activated NF-κB (unmasked p65/RelA) in tumor cells; and (D) negative control of the NF-κB stain by omitting the primary antibody.
IL-1β releases a discrete NF-κB activating signal that is maintained at a higher level by βTRCP. Intriguingly, the elevated βTRCP1 expression in PancTu-1 cells is to some extent related to the autocrine action of IL-1β as shown by the blocking effect of the IL-1 receptor(I) antagonist (Fig. 5A and B). In further support of this notion, exogenously added IL-1β induces βTRCP1 expression in PT45-P1 cells (Fig. 5A and B). Obviously, by inducing βTRCP1 expression, IL-1β contributes to a persistent NF-κB activation that, in turn, maintains IL-1β expression. Even in unstimulated PT45-P1 cells lacking prior IL-1β secretion but exhibiting significant IκBα phosphorylation (i.e., due to an amplified ras/raf1 pathway; Fig. 3A), βTRCP1 overexpression alone is sufficient for inducing constitutive NF-κB activation and cheomoresistance. In this way, βTRCP1 is essentially involved in a potent loop of amplified NF-κB activation, due to a reduced NF-κB repression on the one hand (Figs 2 and 3) and an increased secretion of cytokines such as IL-1β on the other hand (Fig. 5A–C). Thereby, βTRCP1 might participate in the development of cheomoresistance and, possibly, also in pancreatic carcinogenesis.

An involvement of βTRCP in tumorigenesis has been recently proposed for skin, gastric, and colon cancer (39–41). In addition, deregulated βTRCP expression has been linked to other tumor promoting pathways such as down-regulation of the disc large tumor suppressor (Dlg) (ref. 42). The implication of βTRCP1 in tumorigenesis of pancreatic cancer and development of chemoresistance is underscored by the finding that a great number of tumor sections from pancreatic carcinoma patients exhibit increased levels of βTRCP1 expression, as we showed in this study by immunohistochemical analysis of resected tumor tissues. Being reminiscent of the cell lines described above, significant βTRCP1 expression was detected in 65% of the tumor specimens along with strong staining for activated NF-κB (Fig. 6). This was particularly seen in all low differentiated G3 tumors, whereas normal pancreatic tissue did not express βTRCP1 at all. Furthermore, cheomoresistant pancreatic carcinoma cell lines as well as pancreatic ductal adenocarcinoma tissues have recently been shown to express IL-1β quite abundantly (19, 20). Altogether, these findings provide new insights into the molecular mechanism by which pancreatic carcinoma cells develop cheomoresistance in a NF-κB-dependent fashion. As key elements, autocrine IL-1β secretion (i.e., induced by stroma-tumor interactions; ref. 20) and βTRCP1-mediated derepression of NF-κB confer a persistent NF-κB activation and thereby resistance to anticancer drugs.

Acknowledgments

Received 5/10/2004; revised 9/10/2004; accepted 12/10/2004.

Grant support: German Research Society (DFG-Sch677/7-2) and Kiel Medical faculty grant IZK/F89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References


42. Mantovani F, Banks L. Regulation of the discs large tumor suppressor by a phosphorylation-dependent interaction with the β-TrCP ubiquitin ligase receptor. J Biol Chem 2003;278:2477–86.
Increased Expression of the E3-Ubiquitin Ligase Receptor Subunit βTRCP1 Relates to Constitutive Nuclear Factor-κB Activation and Chemoresistance in Pancreatic Carcinoma Cells

Susanne Müerköster, Alexander Arlt, Bence Sipos, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/4/1316

Cited articles
This article cites 40 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/4/1316.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/4/1316.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.