The transcription factor NFκB is a major regulator of genes involved in inflammation and oncogenesis. NFκB is induced upon stimulation of cellular receptors coupled to different intracellular signaling molecules. Further downstream, TRAF6 links at least two receptor pathways to take control of IκB, the administrator of NFκB activity. Here we report on a strong NFκB activation by Tio, a unique herpesviral oncoprotein promoting transformation of human T cells in an Src-kinase-dependent manner. NFκB induction by Tio is independent of Src-kinase interaction and tyrosine phosphorylation of Tio. Mutation of a glutamic acid-rich motif at the N terminus of Tio, corresponding to a TRAF6 consensus binding motif, completely abrogates NFκB activation. Cotransfection of a dominant negative TRAF6 construct led to a decrease in NFκB activation. Furthermore, we provide evidence that TRAF6 directly binds to the Tio oncoprotein. Identification of TRAF6 as the direct target of Tio describes a novel mechanism for the constitutive activation of NFκB through an oncoprotein.

NFκB plays an important role in oncogenesis by promoting genes that have an influence on cell proliferation, cell cycle, differentiation, and apoptosis. It also triggers both innate and adaptive immune responses by transcriptional activation of genes coding for cytokines, chemokines, and adhesion molecules (1, 1–6). Three classical signaling pathways leading to the activation of NFκB are initiated by the stimulation of different receptors. Antigen recognition by the T cell receptor induces NFκB by recruiting protein tyrosine kinases Lck and Zap70 to the receptor complex. The signal is processed by several intermediate proteins, including protein kinase Cθ (PKCθ) and a multimolecular module containing CARMA, Bcl-10, MALT1, and TRAF6. Subsequently, the IkB kinase (IKK) complex and NFκB are activated (5, 7–10). Upon engagement by their cognate ligands, members of the tumor necrosis factor receptor (TNFR) family activate NFκB by recruiting TNFR-associated death domain protein (TRADD) and TNFR-associated factor 2 (TRAF2) (11, 12). The induction of NFκB by the Toll/interleukin-1 receptor family is a prominent event after stimulation with pro-inflammatory cytokines or recognition of pathogen-associated molecular patterns (13). The signal derived from the prototypic Toll-like receptor-4 is dependent on a module containing myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor-associated kinase-1 (IRAK1). The ubiquitin ligase TRAF6 links this module to a trimolecular complex consisting of transforming growth factor-β-activated kinase-1 (TAK1), TAK1-binding protein-1 (TAB1), and TAB2 leading to modification of the IKK complex (14, 15).

The critical step common to these major NFκB-inducing pathways is the activation of the IKK complex. This complex consists of two catalytic subunits, IKK-α (IKK1) and IKK-β (IKK2), and the regulatory subunit IKK-γ (NEMO), which is devoid of kinase activity. In the absence of IKK activity, cytoplasmic IkB binds NFκB and thereby prevents nuclear translocation of this transcription factor. The activated IKK complex phosphorylates IkB, which in turn gets ubiquitinated, dissociates from the NFκB complex, and is degraded by the proteasome. Free NFκB then enters the nucleus and activates transcription (1, 4, 16).

Induction of the transcription factor NFκB is also considered to be important for transformation and immortalization of lymphoid cells by human T cell leukemia virus type-1 or Epstein-Barr virus (2, 17–19). In this context, activation of NFκB is attributed to the viral oncoproteins, the 40-kDa transactivator protein of the pX region (Tax) of human T cell leukemia virus type-1, and the latent membrane protein (LMP)-1 of Epstein-Barr virus, respectively (20, 21). According to the most widely accepted concept, Tax deregulates the IKK complex by linking the scaffold protein IKK-γ to the upstream kinases MEKK1 and NIK, which in turn phosphorylate IKK-α and IKK-β. In addition, Tax supports the oligomerization of IKK-γ to enhance the activity of the IKK complex (22). LMP-1 induces NFκB activity 20–40-fold (19, 23, 24). The signal is transduced by a TNFR-like pathway, where TRADD and TRAF2, which directly interact with LMP-1, are essential (25–27). TRAF6 also affects NFκB activation by LMP-1 through its action downstream of TRADD and TRAF2 (28, 29). An interaction of TRAF6 with TRADD or TRAF2 has not been described for TNFR signaling so far. Thus, Tax activates NFκB by direct modification of the IKK complex, whereas LMP-1 mimics a constitutively active receptor.

**Herpesvirus saimiri** and **Herpesvirus atelis** are members of the γ-herpesviruses or rhadinoviruses. Both induce malignant lymphoproliferation in New World monkeys, except for their natural hosts, the squirrel monkey and the spider monkey, respectively (30–33). Furthermore, **H. saimiri** strain C488 is able to transform human T lymphocytes to permanent growth in culture (34). The saimiri transformation-associated protein of subgroup C (StpC) and the tyrosine kinase-interacting protein (Tip) are the two oncoproteins essential for the T cell-transforming phenotype of strain C488 (35–37). In contrast, the related **H. atelis**
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*tio* encodes only one oncoprotein, the "two-in-one" (Tio) protein. Tio is a membrane-bound protein, forming homodimers or multimers, which exhibits sequence homologies to both StpC and Tip (38). Recombinant *H. saimiri* carrying *tio* in place of *stpC* and *tip* demonstrated that Tio can substitute for StpC and Tip in T cell transformation (39). In previous studies, different analogous functions for Tip and Tio have been reported. They both interact with cellular Src family kinases by binding to their Src homology 3 (SH3) domain (38, 40, 41) and are phosphorylated on distinct tyrosine residues by the bound kinase (42, 43). Tip is phosphorylated on two tyrosine residues, creating binding sites for the SH2 domains of STAT3 and Lck, respectively (43–45). The sole phosphorylation site of Tio, tyrosine residue 136, is essential for the immortalization of primary human T cells (42). A function of Tio related to StpC could not be demonstrated so far. StpC raises the transactivation activity of NFκB 3.5–6-fold. A TRAF2 binding site in the N-terminal region of StpC is essential, not only for TRAF2 binding, but also for NFκB induction and transformation of primary human T lymphocytes (46).

In this report, we describe a novel mode of constitutive NFκB activation by demonstrating a direct interaction between the viral oncoprotein Tio and TRAF6, a key modification enzyme and adapter molecule of multiple NFκB-inducing signaling cascades.

**MATERIALS AND METHODS**

*Cell Culture and Expression Plasmids—* Cultures of transformed human cord blood lymphocytes (CBL-Tio; CBL-M124) were generated as described previously (39). Long term cultures and Jurkat T cells (E6.1; American Type Culture Collection, TIB-152) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), glutamine, and antibiotics. For transfection purposes, Jurkat T cells were cultivated at a maximum concentration of 10⁶ cells/ml.

All mutant Tio plasmids derived from the previously described FLAG-Tio (wild-type Tio, YYYY) construct (38). Deletion mutants were created by PCR amplification with oligonucleotide primers introducing an Nhel recognition sequence, which translates into alanine and serine residues at the deletion site. Mutant D1 was created using primers SH9 (5′-GTTGCTAGCCTTGTACGTCCTGCTCCTC′T-3′) and SH10 (5′-GGTTGCAGCCCGCTGGGACT-3′); mutant D2 with primers SH9 and SH11 (5′-AGCCGCTAGAACAATCCTCAGGCAGCCA-3′); mutant D4 with primers SH14 (5′-CGTGTCT-3′) and SH15 (5′-CAAGCTGTCAGGAGAAGGCAC-3′); mutant D6 with primers SH9 and SH12 (5′-CCATGCTAGAACAATCCTCAGGCAGCCA-3′); mutant D7 with primers SH12 and SH16 (5′-CTTGCTAGCCTGATGTTAGGTG-3′) and SH18 (5′-CAAGCTGTCAGGAGAAGGCAC-3′); mutant D8 with primers SH12 and SH14 (5′-CCATGCTAGAACAATCCTCAGGCAGCCA-3′); mutant D9 with primers SH12 and SH14 (see Fig. 2). Tio point mutants were created by PCR according to the QuikChange® protocol (Stratagene, Heidelberg, Germany). Mutant P1 was created using primers SH17 (5′-GACAAGATGCTACACGC-CCACAAACAACACAAAGAAGAAATCCCTC-3′) and SH18 (5′-GAAGAAGGGTTACCTTGGTTGTTGTTGCTGCTGTTAGCCATCTGTC-3′) to replace Glu-23 and Glu-24 by Gln; mutant P4 using SH23 (5′-GGTTGCAGCCCGCTGGGACT-3′) and SH24 (5′-GGTTGCAGCCCGCTGGGACT-3′) to replace Glu-23 and Glu-24 by Gln; mutant P5 using primers SH17, SH18, SH19, and SH20 to replace Glu-7, Glu-9, and Glu-10 by Gln plus Glu-23 and Glu-24 by Gln; mutant P7 using SH21, SH22, SH23, and SH24 to replace Glu-7, Glu-9, and Glu-10 by Gln plus Glu-23 and Glu-24 by Gln. The mutant PAPA was created using primers PAPA-1 (5′-GAAAC-CTTCTCTGTCCGAGGGAAGCTG-3′) and PAPA-2 (5′-AGT-GCCACACCCCGAGGAAGAGGTTTTT-3′) to change Pro-16 and Pro-17 to Ala, Tio mutant PAQK was created using PAQK-1 (5′-GGCCCACATCAAGACGCAAGACAGAGAGGAGGAGAAGAG-3′) and PAQK-2 (5′-CCTGGCCCTGTTGACCAGAGTCGCCCAGC-3′) to replace Pro-30 to Ala and Gln-31 to Lys; PAPA-2 (5′-CCATGCTAGCCTTGGTTGTTGTTGCTGCTGTTAGCCATCTGTC-3′) and LK-B (5′-CAAGCTGTCAGGAGAAGGCAC-3′) to substitute Leu-18 for Lys within the PAPA mutant. The resulting nucleotide sequences were confirmed by automated sequence analysis on an ABI3100 sequencer (Applied Biosystems, Darmstadt, Germany). Tio mutants FYFF, FYFY, and PARG were described previously (42). Expression plasmids for dominant negative IkBα (IkBoN) and constitutively active IKK2 (IKK2 EE) were kindly provided by R. Vohl (University Erlangen-Nürnberg, Erlangen, Germany) (47, 48). The LMP-1 expression construct was a gift of W. Hammerschmidt (GSF, München, Germany). The TRAF6, the dominant negative TRAF6(300–524), and the TRAF6-HA expression plasmids (28) were kindly provided by A. Kieser (GSF, München, Germany). A plasmid expressing enhanced green fluorescent protein (EGFP), pEGFP-C2, (BD Biosciences) was used to verify transfection efficiency.

*Cell Lysis, Immunoblotting, and Immunoprecipitation—* Jurkat T cells were lysed for 30 min at 4 °C in TNE buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40) containing 5 mM NaF, 1 mM Na3VO4, and 10 μg/ml of each aprotinin and leupeptin (Sigma). Lysates were cleared at 13,000 revolutions/min for 10 min. Protein concentrations were determined by BCA assay (Pierce), and equal amounts of protein were separated by denaturating SDS-PAGE. Polyvinylidene difluoride membrane filters (Amersham Biosciences) were used for immunoblotting and incubated with antisera against Tio (38), TRAF6 (H-274; Santa Cruz Biotechnology; diluted 1/1000), TRAF2 (C-20; Santa Cruz, Biotechnology; diluted 1/1000), or with monoclonal antibodies directed against β-actin (AC-15; Abcam, Cambridge, UK; diluted 1/2000), α-FLAG (anti-FLAG® M2-heraldseh peroxidase; Sigma; diluted 1/2000), or α-HA (HA.11; Covance, Princeton, New Jersey; diluted 1/1000), diluted in phosphate-buffered saline, pH 7.4, containing 0.1% Tween 20 and 5% milk powder for 12 h at 4 °C. For detection of primary antibodies, horseradish peroxidase-conjugated antibodies, either against mouse (F(ab)2; Amersham Biosciences) or rabbit (Dako, Hamburg, Germany; diluted 1/2000) immunoglobulins were used. Immunodetection was performed by enhanced chemiluminescence according to the manufacturer’s protocol (Amersham Biosciences). For immunoprecipitation, 500 μl of cleared lysates (800–1300 μg of protein) were mixed with antisera against TRAF6, TRAF2 (2 μg of each), or Tio (2 μl) and rotated at 4 °C overnight. The next day, 20 μl of protein A-Sepharose (Sepharose CL-4B; Amersham Biosciences) were added for 1 h at 4 °C. FLAG-tagged proteins were incubated with 20 μl of anti-FLAG-agarose (Sigma) at 4 °C overnight. The beads were washed five times with lysis buffer. Samples were boiled for 5 min in 20 μl of SDS-loading buffer and applied to SDS-PAGE.

**Transfection—** Ten million Jurkat T cells per sample were electroporated with 25–100 μg of DNA. Vector plasmid (pcDNA3) was used to equalize promoter copy numbers in all transfections. Electroporation
was carried out with an Easyject Plus apparatus (Equibio, Boughton, UK) at 250 V, 1500 microfarads. The cells were transfected in complete medium without antibiotics, harvested 48 h post-transfection and washed in phosphate-buffered saline. Two-thirds of the cells were used for Western blot analysis, and one-third was used for luciferase reporter assays.

**Reporter Plasmids and Assays**—The reporter construct pNFκB-Luc contains five NFκB binding sites in front of a luciferase gene (Stratagene, Heidelberg, Germany). Experimental integrity was confirmed with alternative NFκB reporter constructs, pBLIkLuc with four NFκB binding sites (49) and its mutant pBL-SK (48). In addition, AP-1 or NFAT luciferase reporter constructs (50) were tested. Jurkat T cells were transfected with expression plasmids and 8 μg of reporter DNA. Cell pellets were resuspended in 300 μl of lysis buffer (25 mM Tris-HCl, pH 7.8, 2 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 2 mM DCTA (trans-1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid monohydrate; Sigma) and incubated at room temperature for 30 min. Lysates were cleared by centrifugation for 5 min at 13,000 revolutions/min. Assay buffer (100 μl; 100 mM K3PO4, 15 mM MgSO4, 5 mM ATP) was mixed with lysates (25 or 50 μl) in a 96-well plate. Assay buffer (100 μl) supplemented with β-luciferin (1 mM) (Roche Applied Science) was added, and luciferase activity was determined with a luminometer (Orion, Berthold, Germany). Relative luciferase activity was calculated by dividing the sample values by the value of vector DNA (pcDNA3) cotransfected with the reporter construct.

**Drug Treatment of Transfected Cells**—Transfected Jurkat T cells were dispensed into 12 wells 24 h post-electroporation and treated for 24 h with PMA (Sigma), ionomycin (Sigma), PKC inhibitors Gö6983 (51), or Ro-31–8220 (Calbiochem, Merck; Schwabach, Germany), respectively (52), TNFα (ImmunoTools, Friesoythe, Germany), or the TNFα inhibitor Remicade® (Infliximab, Essex Pharma, München, Germany), as indicated in the legend to Fig. 4. The volume of lysis buffer was reduced to 100 μl/sample.

**Subcellular Fractionation of Proteins**—Transformed human cord blood lymphocytes were harvested by low speed centrifugation at 500 × g for 5 min. The pellet was resuspended in hypotonic lysis buffer (20 mM Tris, pH 7.5, 5 mM EDTA, protease inhibitors), and 30 strokes were delivered to the cell suspension in a tight-fitting Dounce homogenizer. Cell nuclei were removed by centrifugation at 4,000 × g for 15 min. Cellular membranes were sedimented from the remaining supernatant at 20,000 × g for 30 min. The final supernatant containing cytosol and free lipid was supplemented by adding an equal volume of 2-fold-concentrated TNE buffer including protease inhibitors. Pellets containing cellular membranes were dissolved in TNE buffer, and insoluble constituents were removed by centrifugation at 20,000 × g for 10 min. Protein concentrations were determined and equal amounts were analyzed by Western blot or immunoprecipitation.

**RESULTS**

**NFκB Activity Is Induced by the Tio Oncoprotein in T Cells**—NFκB activation is an important function of distinct viral oncoproteins, such as LMP-1, Tax, or StpC (21, 23, 24, 46, 53, 54). Here we have investigated whether Tio also affects the activation of NFκB. After cotransfection of Tio expression constructs with NFκB-specific reporter plasmids into Jurkat T cells, relative luciferase activity was measured. The expression of the Tio oncoprotein was confirmed by Western blot analysis. The strong induction of NFκB activity observed was directly proportional to the amount of Tio-DNA transfected (Fig. 1). Significant NFκB activa-
tion could be measured for amounts of Tio far below the detection limit of the immunoblot (Fig. 1). NFκB induction by Tio was confirmed with an alternative NFκB luciferase reporter construct. In contrast, Tio did not stimulate luciferase expression from reporter plasmids with either mutated NFκB binding sites or consensus AP-1 or NFAT response elements, respectively (data not shown). NFκB activation by Tio was blocked when dominant negative IkBα (IkBα DN) was cotransfected (Fig. 1). Suppression of NFκB activation also reduced the expression of Tio, which is most likely explained by the positive regulatory effect of NFκB on the cytomegalovirus promoter (55, 56). Increased amounts of Tio plasmid augmented the expression level but did not reconstitute NFκB activity in the presence of IkBα DN (Fig. 1). This demonstrated that Tio acts upstream of IkBα to induce NFκB.

**NFκB Activation by Tio Is Independent of Src-Kinase Interaction**—Src interaction and phosphorylation of Tyr-136 of Tio is essential for the transformation of human T lymphocytes in vitro (Fig. 2) (42). Tio mutants FYFF, YFY, and PARG were tested for their ability to activate NFκB. The mutants PARG and YFY, which were deficient for phosphorylation of Tyr-136, and control mutant FYFF, which acts like wild-type Tio in Src-kinase interaction and T cell transformation, did not differ in their ability to induce NFκB activation (Fig. 3). Thus, NFκB activation by Tio was independent of Src-kinase interaction and Tyr-136. These mutants were further used as positive controls in the reporter assays. LMP-1 of Epstein-Barr virus (20) and *H. saimiri* StpC/Tip were included as additional positive controls. Transfections with empty vector DNA or an expression construct for EGFP served as negative and transfection efficiency controls, respectively.

**Identification of Tio Elements Required for NFκB Induction**—NFκB activation by other viral oncoproteins is based on specific protein-protein interactions with upstream regulators. The interaction of TRAF2 with a consensus binding motif in StpC was shown to be essential for the activation of NFκB (46). For identification of the region in Tio substantial for NFκB activation, sequence comparisons were performed between Tio, StpC, LMP-1, and other TRAF2-interacting proteins. The TRAF2 consensus binding sequence has been reported to be P(X)Q(X)(T/S) (58), which is common among TRAF1, -2, -3, and -5 but differs from that of TRAF6 (59). StpC contains the motif PIEET, an incomplete consensus motif still binding to TRAF2. We identified two related motifs in Tio, PLGDS, and PQDPT, which were targeted for mutation of the important first proline residue (Fig. 2). The resulting Tio mutants PAPA, PAPALK, and PAQK induced NFκB activity in a similar manner as the wild-type protein (Fig. 3 and data not shown). In addition, coimmunoprecipitation analysis failed to demonstrate an
interaction of TRAF2 with Tio (data not shown). Thus, TRAF2 does not seem to be involved in Tio signaling to NFκB.

To narrow down a suspected domain responsible for NFκB activation by Tio, deletion mutants in the StpC homologous part were created (Fig. 2, D1, D2, D4, D6–D8). All deletion mutants, except mutant D7, failed to induce NFκB activity (Fig. 3A), indicating that the required region is located in the N-terminal segment of Tio. Within this segment, two glutamic acid-rich motifs were found. Acidic amino acids forming a region of net negative charge were shown to be important for protein-protein interactions, like it was demonstrated for StpC (60). Mutation of glutamic acids Glu-7, Glu-9, and Glu-10 and/or Glu-23 and Glu-24 in Tio to glutamine (Gln) or glycine (Gly) resulted in Tio mutants P1–P5 and P7 (Fig. 2). NFκB reporter assays indicated that mutation of Glu-23 and Glu-24 to Gln or Gly (P2, P4) had no impact on NFκB induction. In contrast, mutation of Glu-7, Glu-9, and Glu-10 to Gln or Gly (P1, P3, P5, P7) abrogated NFκB activation (Fig. 3, B and C). Western blot analysis revealed reduced expression levels of all NFκB negative mutants, which were again attributed to the NFκB sensitivity of the cytomegalovirus promoter. Expression of these Tio mutants was augmented by increasing the amount of plasmid transfected. However, this did not correlate with an increase of luciferase activity in the reporter assay (Fig. 3, A–C). Deletion mutants and point mutants with a conservative exchange of glutamic acid (Glu) against glutamine (Gln) were retested in triplicates, and the S.D. was calculated (Fig. 3D). These experiments confirmed that NFκB activation depends on glutamic acid residues at the N terminus of Tio.

**Tio Does Not Utilize TNFR or PKC-dependent Pathways for NFκB Activation**—NFκB-activating signaling pathways in T cells may originate from the T cell receptor, the TNFR, or the toll-like receptors. To test for the influence of Tio on TNFR-mediated NFκB activation, transfected cells were stimulated with different amounts of TNFα. NFκB activation by Tio appeared not to be affected by increasing amounts of TNFα. However, TNFα induced a dose-dependent rise of NFκB activity in cells transfected with the vector-DNA, which served as a negative control, as well as with P1 mutant (Fig. 4). To exclude TNFR activation by Tio- or transfection-induced TNFα expression, a potent TNFα inhibitor (Remicade) was added to transfected Jurkat T cells. There was no difference between treated and untreated cells. To control whether Remicade efficiently inhibits TNFα signaling, transfected cells were treated with TNFα and the inhibitor simultaneously. This treatment prevented the dose-dependent rise of NFκB activity by TNFα (Fig. 4).

A key effector of the T cell receptor pathway is PKCθ. To investigate whether Tio acts upstream or at the level of PKCθ to induce NFκB, we applied PKC inhibitors to transiently transfected Jurkat T cells. The inhibitors Go6983 and Ro-31–8220 are specific for different PKC isoenzymes, among them PKCθ. In the NFκB reporter assay, no difference was observed between untreated T cells and samples exposed to Go6983 or Ro-31–8220 (Fig. 4). To guarantee the functionality of the PKC inhibitors, cells were stimulated with PMA and ionomycin to mimic NFκB activation via the T cell receptor pathway. The induction of NFκB by these drugs in all transfected samples was efficiently suppressed by the simultaneous addition of Go6983 or Ro-31–8220 (Fig. 4). With these assays, we suggest that the signal for NFκB activation by Tio is not mediated by components of the TNFR or the PKC-dependent pathway.

**TRA6(300–524) Blocks NFκB Activation by Tio**—TRA6 is an essential mediator in the NFκB signaling cascades triggered by various receptors and oncoproteins. Mutational analysis of Tio revealed the functional relevance of glutamic acid residues at the N terminus, which are major constituents of a TRA6 consensus binding motif (PXEXX(Ar/Ac)) (Ar, any aromatic residue; Ac, any acidic residue) (59)
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FIGURE 6. TRAF6 directly interacts with Tio at the consensus TRAF6 binding motif. Jurkat T cells were transiently transfected with expression vector DNA or plasmids encoding HA-tagged TRAF6 and FLAG-tagged Tio or mutant P1 in the combinations given on top of the figure. In an additional set of transfections, constitutively active IKK2 (IKK2 EE) was cotransfected to override the influence of NFκB on transcription from expression vectors (column to the right). Proteins were immunoprecipitated with either anti-FLAG monoclonal antibody (IP FLAG) or a polyclonal TRAF6 antibody (IP TRAF6). Control precipitations without antibodies (Ø Ab) were included with each experiment. Immune complexes and cell lysates (C) were assayed for the presence of FLAG-Tio (FLAG), HA-TRAF6 (HA), or total TRAF6 (TRAF6) with the respective antibodies (shown on the left). hc, immunoglobulin heavy chain.

(Fig. 2). Therefore, we overexpressed TRAF6 or dominant negative TRAF6(300–524) in Jurkat T cells and analyzed their effect on Tio-mediated NFκB activation. TRAF6 or TRAF6(300–524) alone induced no NFκB activity compared with vector-transfected cells. Furthermore, TRAF6 did not influence the Tio-induced activity. However, cotransfection of TRAF6(300–524) caused a dramatic decrease of Tio-induced NFκB activity (Fig. 5). This indicates that TRAF6 is a major factor transmitting the stimulatory signal from Tio to NFκB.

Tio Binds to TRAF6—Because of the impact of dominant negative TRAF6(300–524) on NFκB activity by Tio and the essential role of a consensus TRAF6 binding motif in Tio, we supposed a direct interaction between TRAF6 and Tio. To examine this hypothesis, we performed coimmunoprecipitation analyses. Interaction of Tio with TRAF6 at the predicted TRAF6 binding motif PQEHEE was controlled by Tio mutant P1, where the essential glutamic acid residues were mutated to glutamine. Jurkat T cells were cotransfected with combinations of HA-tagged TRAF6 and FLAG-tagged Tio or P1, respectively. Because expression levels of the cytomegalovirus promoter-driven constructs appeared to be regulated by NFκB, we complemented our experiment by cotransfection of constitutively active IKK2 (IKK2 EE) to ensure comparable expression levels of Tio and P1. Expression from the transfected plasmids was controlled by Western blot analysis of cell lysates using adequate antibodies (Fig. 6C).

FIGURE 7. Membrane recruitment and Tio interaction of TRAF6 in transformed human T cells. A, cytoplasmic (c) and membrane (mem) proteins of transformed lymphocytes expressing either Tio (CBL-Tio) or StpC/Tip (CBL-M124) were separated and assayed for the presence of TRAF6 and Lck. Whole cell lysate (wcl) of Jurkat T cells served as a positive detection control. B and C, soluble proteins from membrane fractions of transformed T cells were subjected to immunoprecipitation with either anti-FLAG monoclonal antibody (IP FLAG) or a polyclonal TRAF6 antibody (IP TRAF6). Coprecipitated Tio or TRAF6 was detected with the corresponding antibodies (shown on the left).

Tio and P1 were immunoprecipitated using anti-FLAG-agarose (Fig. 6A). Presence of the precipitated proteins (Tio and P1) was verified with an anti-FLAG antibody. Coprecipitated TRAF6 was detected by HA monoclonal antibody only from samples cotransfected with Tio and TRAF6. The polyclonal TRAF6 antiserum detected both endogenous and exogenous TRAF6. Tio mutant P1 did not bind any TRAF6. Cotransfection of IKK2 EE provided higher expression levels of mutant P1 but gave the same results. In the reverse experiment, the TRAF6 polyclonal antibody was used for immunoadsorption leading to precipitation of exogenous, as well as endogenous, TRAF6 proteins (Fig. 6B). Western blot analysis of TRAF6 complexes with anti-FLAG antibody established the interaction of Tio with TRAF6. In contrast, mutant P1 was not able to bind to TRAF6. These experiments defined the motif PQEHEE of Tio, which corresponds to a TRAF6 consensus binding sequence, as a direct interaction site for TRAF6.

Interaction of TRAF6 with Tio in Virus-transformed Human T Cells—We next wanted to test for the association of TRAF6 and Tio in human T cells transformed by recombinant H. saimiri C488. Because of low expression levels, TRAF6 precipitation from post-nuclear lysates was not suitable to detect binding to Tio (data not shown). In an attempt to enrich TRAF6, we extracted cytosolic and membrane proteins from virus-transformed lymphocytes expressing either StpC/Tip or Tio and analyzed these fractions by Western blot. Enrichment of the protein tyrosine kinase Lck in the membrane preparations confirmed the integrity of fractionation. In the presence of Tio, the majority of TRAF6 was recruited to the membrane fraction, whereas TRAF6 was mainly cytoplasmic in T cells expressing StpC and Tip. (Fig. 7A). Immunoprecipitation analyses with these solubilized membrane preparations revealed binding of TRAF6 to Tio (Fig. 7B). These results demonstrated that Tio interacts with TRAF6 and thereby recruits this cellular regulator to the membrane fraction of T cells transformed by recombinant H. saimiri.
LMP-1 also engages TRAF6 for NFκB virus that is involved in several forms of human malignancies (61). TRAF6 distinguishes Tio from LMP-1, an oncoprotein of Epstein-Barr oncoproteins directly address signaling intermediates common to different interactions with upstream regulators TRADD and TRAF2 (25, 26, 28, 29). Stable multimers of LMP-1 are localized in the plasma membrane and are considered to form constitutively active signaling complexes related to those induced by activated members of the TNFR family, which also utilize TRADD and TRAF2 to induce growth-promoting signals via NFκB (11, 12, 62, 63). A similar model of receptor mimicry has been established to explain the oncogenic properties of H. saimiri StpC (46, 64). Tio also forms oligo- or multimers, and its primary amino acid sequence suggests membrane localization (38). Therefore, Tio likely induces TRAF6-containing signaling complexes that are without precedent among known membrane receptors or viral oncoproteins. However, Tio-TRAF6 binding is reminiscent of the interaction between human T cell leukemia virus type-1 Tax and IKK-β (29). Stable multimers of LMP-1 are localized in the plasma membrane but dispensable for Tio signaling to NFκB (11, 62–65). A major question arising from these observations concerns the composition and localization of Tio-induced protein complexes. Tio-TRAF6 and Tio-Src complexes may represent specific signaling entities segregated into different cellular compartments. However, the organization of the binding sites on Tio would also allow for the simultaneous binding of both Src kinases and TRAF6. Due to the modular structure of the proteins involved, additional binding partners are likely to be recruited into the complex(es). With respect to NFκB induction, inclusion or exclusion of known regulators and effectors of TRAF6 will be of special interest. So far, TRAF2 may be excluded, as mutation of suspected TRAF2 binding sites did not alter the NFκB-inducing ability of Tio (Figs. 2 and 3, mutants PAPA, PAQK, and PAPALK and data not shown) and no binding to Tio was detected (data not shown). In Toll-like receptor-4 signaling, TRAF6 directly binds to interleukin-1 receptor-associated kinase-1 (70), and further downstream a complex is formed by TAB1, TAB2, TAK1, and TRAF6 (14). In addition to TAK1, the MAPKKKs, NIK, and MEKK1 were reported to activate IKK (71, 72). In this context, Tio might act as a scaffold and induce or stabilize protein interactions that facilitate IKK phosphorylation and thus NFκB activation.

Another open question addresses the biological relevance of TRAF6 interaction and NFκB activation in T cell transformation and oncogenesis by viruses expressing Tio. NFκB is a ubiquitous transcription factor involved in numerous cellular events leading to cell proliferation and/or apoptosis inhibition (2, 3). Thus, it is conceivable that Tio, similar to Tax, LMP-1, and StpC, exploits this pathway to exert its oncogenic potential. Further studies will be required to analyze the role of NFκB activation and Tio-TRAF6 interaction in the promotion of T cell growth and tumor induction, progression, and maintenance.

Acknowledgments—We thank Arnd Kieser, Wolfgang Hammerschmidt, and Reinhard Voll for providing expression constructs.

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