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Human T-cell leukemia virus type I Tax-protein-mediated activation of NF-κB from p100 (NF-κB2)-inhibited cytoplasmic reservoirs

TOMOHIKO KANNO, GUIDO FRANZOSO†, AND ULRICH SIEBENLIST‡

Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **The human T-cell leukemia virus type I Tax protein transforms T cells through induced expression of many cellular genes, including those encoding the growth-related proteins interleukin 2 and the α chain of its receptor. Induction of these genes is mediated, at least in part, through Tax-dependent posttranslational activation of NF-κB, typically heterodimers of p50 (NF-κB1) and p65 (RelA). The preexisting NF-κB proteins are retained in the cytoplasm of cells by association with inhibitory ankyrin-motif-containing IκB proteins, primarily IκB-α but also including the precursor proteins p105 (NF-κB1) and p100 (NF-κB2). Here we demonstrate the existence of a previously undescribed multimeric cytoplasmic complex in which NF-κB dimers are associated with the p100 inhibitor in a manner dependent on the precursor protein's ankyrin domain. We also demonstrate an antagonistic effect of the Tax protein on the cytoplasmic sequestration function of p100; this in turn leads to nuclear translocation of NF-κB dimers liberated from multimeric complexes. Tax may exert these effects through the physical association with p100. Tax also relieves the p100-mediated inhibition of DNA binding by p50–p65 heterodimers *in vitro*. The results demonstrate a mechanism by which Tax may activate NF-κB in T cells.**

The human T-cell leukemia virus type I (HTLV-I) is the etiologic agent causing adult T-cell leukemia and tropical spastic paraparesis. These pathogenic consequences of viral infections are closely associated with the expression of the viral *tax* gene (for review, see ref. 1). Tax protein induces transcription of many cellular genes including, among others, interleukin 2 and interleukin 2 receptor α genes and c-*fos*; products of such genes are likely to mediate Tax-protein-induced cellular transformation of T cells. Tax also induces transcription of viral genomes including that of human immunodeficiency virus type 1 and HTLV-I. Tax exerts these activities through distinct cis-acting regulatory DNA elements, including the cAMP response element (CRE), the serum response element, and NF-κB binding sites.

Tax-mediated transactivation through κB binding sites depends on translocation of NF-κB from the cytoplasm into the nucleus, as demonstrated in T cells carrying an inducible expression vector for Tax (2). In the absence of specific signals, NF-κB dimers are normally kept in the cytoplasm by association with their primary inhibitor, IκB-α; in addition, the precursor proteins p105 (NF-κB1, the precursor of p50) and p100 [NF-κB2, the precursor of p52 (also p50B, p49, or lyt-10)] have been implicated to function as inhibitors due to the presence of ankyrin domains in their C-terminal halves (refs. 3–7; for review, see ref. 8). Activation of NF-κB by Tax thus suggests a cytoplasmic role for this viral protein, consistent with its presence in both nuclear and cytoplasmic compartments (9). Kinetically, Tax first activates primarily p50–p65 heterodimers from preexisting cytoplasmic reservoirs (2). Thereafter, and likely as a consequence of this early activation, c-Rel-containing complexes accumulate in the nucleus (2), and such complexes are frequently observed in HTLV-I-infected cells (10).

Here we describe a mechanism by which Tax may activate NF-κB p50–p65 dimers to translocate to the nucleus. Tax antagonizes the cytoplasmic sequestration function of p100, mediated most likely through its strong association with p100 (ref. 11; see also this paper). Surprisingly, multimeric complexes containing NF-κB dimers in association with p100 are the targets of this Tax activity.

MATERIALS AND METHODS

Cells, Transfection, Whole-Cell Extract Preparation, and Immunofluorescence. JPX-9 and JPX/M cells (12) were incubated with 120 μM $ZnCl_2$ for 12 h to induce the functional Tax and a mutant Tax protein, respectively. The human embryonic cell line NTera-2 cells, the pMT2T series of expression vectors, and the NTera-2 transfection protocol have been described (13–16). pMT2T-p65* contains a cDNA clone for human p65 (17) truncated at the *Bsp*HI site, just C-terminal to the nuclear localization signal. The truncated protein dimerizes with p50 and can be inhibited by IκB-α but lacks transactivating ability in chloramphenicol acetyltransferase assays (ref. 18 and G.F. and U.S., unpublished observations). pMT2T-Tax encodes a full-length Tax protein (19). The pMT2T-p100N vector was previously described as pMT2T-p50B (13, 15). The total amount of transfected DNA was kept constant by adding appropriate amounts of the insertless pMT2T vector. Whole-cell extracts were prepared by repeated freezing–thawing as described (14). Indirect immunofluorescence was performed on transfected NTera-2 cells as described (15).

Immunoprecipitation (IP). Whole-cell extracts were immunoprecipitated with appropriate antibodies and protein A-Sepharose beads in 50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% Nonidet P-40/1 mM EDTA. After intensive washing, the antigen–antibody complexes were separated by SDS/PAGE and examined by the ECL Western blot analysis (Amersham) using appropriate antibodies.

Double IP. Whole-cell extracts were immunoprecipitated with anti-p50-peptide antibody (14, 15) and protein A-Sepharose beads in the buffer described above. After intensive washing, the antigen–antibody complexes were eluted with an excess (6 μg of peptide per μl of antibody) of the specific peptide for 16 h at 4°C. The resulting supernatants were immunoprecipitated with anti-p65-peptide antibody (14) and protein A-Sepharose beads. The final immune complexes

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Abbreviations: RHD, Rel/NF-κB homology domain; IP, immunoprecipitation; EMSA, electrophoretic mobility shift assay; HTLV-I, human T-cell leukemia virus type I; CRE, cAMP response element; NRS, normal rabbit serum.

†Permanent address: Servizio di Microbiologia del Complesso Convenzionato Università-Ospedale, Via Ospedale 2, 35100 Padua, Italy.

‡To whom reprint requests should be addressed at: Building 10, Room 11B-16, National Institutes of Health, Bethesda, MD 20892.

were separated by SDS/PAGE and examined by Western blot analysis with anti-p100 monoclonal antibody (15).

Electrophoretic Mobility Shift Assay (EMSA). Whole-cell extracts were incubated for 30 min with a ^{32}P-labeled κB palindromic probe (13) in 0.1 M KCl/10 mM Hepes, pH 7.5/0.2 mM EDTA/poly(dI-dC) (0.1 μg/ml) (Pharmacia)/bovine serum albumin (1 μg/ml)/0.5 mM dithiothreitol. The protein–DNA complexes were analyzed as described (13).

Antibodies. The antibodies used were an anti-p50-peptide antibody (14, 15), an anti-p65-peptide antibody (14), an anti-p65 rabbit polyclonal antibody (15, 16), an anti-Tax (C-terminal peptide) rabbit antibody (kindly provided by J. N. Brady, National Institutes of Health, Bethesda), an anti-p100N rabbit polyclonal antibody (15), an anti-p100 monoclonal antibody that recognizes an epitope in the N terminus (15), and an anti-IκB-α rabbit antibody (16).

RESULTS

The activation of NF-κB by Tax involves the release of preformed NF-κB complexes from cytoplasmic stores (2). To explore potential underlying molecular mechanisms, we asked whether the previously reported association of Tax with the precursor proteins p100 (NF-κB2) (11) and p105 (NF-κB1) (20) might play a role in the activation process. We confirmed the reported association of Tax with the N-terminal half of p100 (equivalent to the p52 mature processed form of p100) (11) by co-IP out of appropriately transfected NTera-2 cells (data not shown); NTera-2 embryonal carcinoma cells are devoid of endogenous NF-κB activity and, therefore, ideal for this analysis (14, 15). By comparison, the association of Tax with the C-terminal half of p100 or with p105 was negligible, suggesting a much lower affinity of Tax for these latter proteins, in agreement with Beraud *et al.* (11). Because of these data, we investigated whether Tax has an effect on the inhibitory function of the ankyrin-containing p100 protein.

p100 can dimerize with p65 and c-Rel through the Rel/NF-κB homology domains (RHD; for review, see ref. 8) and retain these proteins in the cytoplasm (3–6). We transfected NTera-2 cells with expression vectors for p50, p65*, p100, and Tax to investigate whether Tax affects the cytoplasmic sequestration function of p100 (p65* lacks transactivation domains to avoid induction of endogenous IκB-α; see ref. 16). p65* was exclusively nuclear when coexpressed with only p50 (Fig. 1*A*) but was almost completely sequestered in the cytoplasm when coexpressed with p100 (Fig. 1*B*). However, coexpression of Tax, in addition, resulted in significant nuclear localization of p65*, despite the presence of p100, although we consistently observed that a portion of p65* remained in the cytoplasm (Fig. 1*C*). Because p100 was cytoplasmic regardless of the presence of Tax (Fig. 1 *E* and *F*), Tax may have dissociated p65* from p100 to effect its nuclear translocation. Qualitatively similar results were also obtained with full-length p65 (data not shown). Tax was both cytoplasmic and nuclear (Fig. 1*D*).

p100–p65 heterodimers are retained in the cytoplasm. We therefore considered that in the above experiment p65 may have preferentially dimerized with p100 rather than with p50 and that Tax may then have disrupted these heterodimers to effect the nuclear translocation of p65. Consistent with the formation of p100–p65 heterodimers, the amount of p50–p65 heterodimers decreased in the presence of p100, as judged by coIP (Fig. 2*A*, compare lanes 1 and 2). However, the additional presence of Tax did not effect these associations; the amount of p50–p65 heterodimers was not restored to the levels seen in the absence of p100 with Tax, even with higher Tax concentrations (Fig. 2*A*, lane 3, and data not shown). This argues against a Tax-mediated dissociation of p100–p65 heterodimers. The existence of such Tax-insensitive heterodimers most likely explains why Tax did not cause all of p65 to translocate to the nucleus.

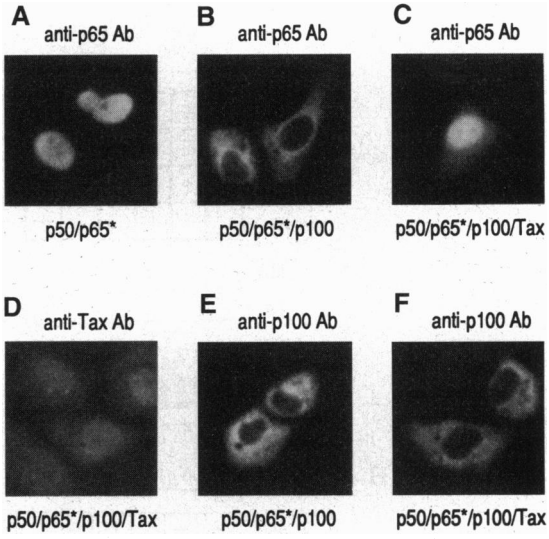

FIG. 1. Tax protein decreases the cytoplasmic sequestration of p65 by p100 in NTera-2 cells that were transfected with expression vectors for the NF-κB components as indicated. (*A*) pMT2T-p50 (1 μg)/pMT2T-p65* (1 μg). (*B* and *E*) pMT2T-p50 (1 μg)/pMT2T-p65* (1 μg)/pMT2T-p100 (2 μg). (*C*, *D*, and *F*) pMT2T-p50 (1 μg)/pMT2T-p65* (1 μg)/pMT2T-p100 (2 μg)/pMT2T-Tax (2 μg). Indirect immunofluorescence analysis was performed with the antibodies (Ab) shown.

Importantly, a significant portion of p65 was apparently not dimerized with p100 but, rather, remained associated with p50 in these experiments (Fig. 2*A*, lane 2); nevertheless, this portion of p65 was also retained in the cytoplasm by p100 (Fig. 1). We considered the possibility that p100 may form multimeric complexes in the cytoplasm with classical p50–p65 heterodimers and that such multimeric complexes may be targets for Tax. The ankyrin-containing C-terminal half of p100 could have interacted with p50–p65 dimers, shielding their nuclear localization signals. To test this, we established a sequential coIP (double IP) protocol. Whole-cell extracts from p50–p65–p100-transfected NTera-2 cells were immunoprecipitated first with anti-p50 antibodies, eluted with cognate p50 peptide, and then reimmunoprecipitated with anti-p65 antibodies; this procedure coimmunoprecipitated p100, as shown by Western blot analysis (Fig. 2*B*, lane 1), demonstrating a multimeric association. In control experiments, expression of either p50 or p65, but not both (Fig. 2*B*, lanes 2 and 3), or of the N-terminal half of p100 (p100N) instead of p100 (Fig. 2*C*, lane 2) did not result in coIP of either p100 or p100N, respectively (see also Figs. 2*B* and 3*D* for p52, the naturally processed form of p100). Therefore, a multimeric complex including p50, p65, and p100 existed *in vivo* and required the C-terminal ankyrin-containing half of p100 for association. In support of this view, the coexpression of the C-terminal half of p100 as a separate protein effectively competed with p100 for association with p50–p65 heterodimers (data not shown). Importantly, these multimeric complexes were detected also in untreated Jurkat T cells and JPX-9 Jurkat T cells (Fig. 2*D*, lane 1, and data not shown). The stringency of the assay was further confirmed by replacing the first or the second antibodies with either preimmune serum (NRS) or an irrelevant antibody, anti-IκB-α (Fig. 2*D*, lanes 2, 3, 5, and 6). [A fraction of the complexes precipitated with anti-p50 antibodies was spontaneously released even without the cognate peptide (compare lanes 1 and 4).] Double IP experiments performed as above but with extracts from metabolically labeled cells indicated that a significant portion of p50–p65 is associated with p100 as compared with that associated with IκB-α (data not shown). However, absolute

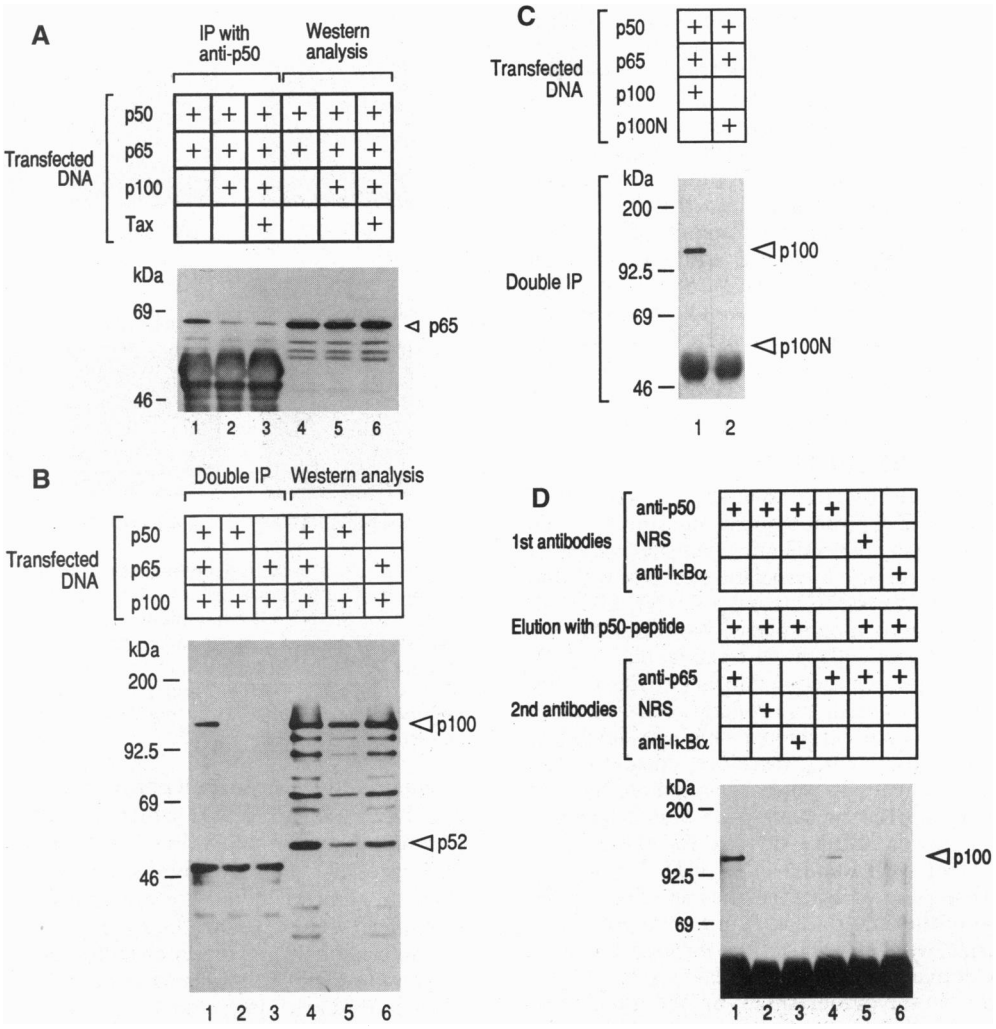

FIG. 2. Multimeric complexes including p50, p65, and p100 exist in transfected NTera-2 cells and in Jurkat T cells. (*A*) Tax does not affect RHD-mediated dimerization. NTera-2 cells were transfected with pMT2T-p50 (1.5 μg), pMT2T-p65 (1.5 μg), pMT2T-p100 (3 μg), or pMT2T-Tax (3 μg) in combinations as indicated. Whole-cell extracts were separated by SDS/PAGE either after IP with anti-p50 antibody (lanes 1–3) or directly (lanes 4–6). Western blot analysis was performed with anti-p65 antibody. (*B*) NTera-2 cells were transfected with pMT2T-p50 (3 μg), pMT2T-p65 (3 μg), or pMT2T-p100 (3 μg) in combinations as indicated. Whole-cell extracts (60 μg) were subjected to the double IP procedure. The final immune complexes (lanes 1–3) and the original whole-cell extracts (15 μg; lanes 4–6) were examined by Western blot analysis using anti-p100 monoclonal antibody. The positions of p100 and its processed form p52 are indicated. The ≈50-kDa band in lanes 1–3 is due to the immunoprecipitating antibody. (*C*) NTera-2 cells were transfected with expression vectors (each at 2 μg) in combinations as indicated. The double IP procedure was performed. (*D*) The double IP procedure was performed with whole-cell extracts (150 μg) from untreated JPX-9 Jurkat T cells with the experimental conditions modified as indicated. Lanes: 1, with the standard procedure without modification; 2 and 3, the antibody for the second IP was replaced with the preimmune normal rabbit serum (NRS) or an irrelevant anti-IκBα antibody, respectively; 4, the step for elution was performed without adding the cognate p50 peptide; 5 and 6, the antibody for the first IP was replaced with NRS or the irrelevant antibody, respectively.

amounts of the different pools of cytoplasmic NF-κB remain to be determined.

The multimeric cytoplasmic complexes containing p100 and p50–p65 heterodimers are targets for Tax. While p50–p65 dimerization was not affected by Tax (Fig. 2*A*, lane 3), the amount of the multimeric complexes was significantly decreased by coexpression of Tax, as estimated by the amount of p100 coimmunoprecipitated with the double IP protocol (Fig. 3*A*, lanes 1 and 2; 91% decrease by densitometry). At the same time, physical interaction between Tax and p100 was observed (Fig. 3*B*; a nonspecific background band migrates closely to Tax but can be differentiated from it). Therefore, Tax dissociates multimeric complexes, perhaps via direct physical interaction with p100, thus liberating p50–p65 heterodimers from inhibition. The same results were obtained with the truncated p65* (data not shown). This provides an explanation for the Tax-mediated nuclear translocation of p65 observed in Fig. 1.

To confirm these observations and to assess their physiological relevance, we examined another system. JPX-9 and JPX/M cells are Jurkat cells stably transfected with plasmids encoding wild-type Tax and mutant Tax proteins, respectively; these Tax proteins are inducible by $ZnCl_2$ (12). NF-κB was induced by $ZnCl_2$ in JPX-9 cells but not in JPX/M cells (Fig. 3*C*). We have shown (2) that the initial NF-κB complexes activated by Tax are primarily p50–p65 heterodimers, which are released from preformed cytoplasmic pools. In JPX-9 cells, Tax caused a significant decrease in the amount of p100 coimmunoprecipitated by the double IP protocol (Fig. 3*D*, lanes 1 and 2; 66% decrease by densitometry), which indicates a Tax-mediated release of p50–p65 heterodimers. In contrast, no corresponding change was observed with mutant Tax in JPX/M cells (Fig. 3*D*, lanes 5 and 6). Finally, the physical interaction between Tax and p100 was also detected in JPX-9 cells (Fig. 3*E*). Therefore, Tax dissociated p100 from naturally existing multimeric complexes in Jurkat cells

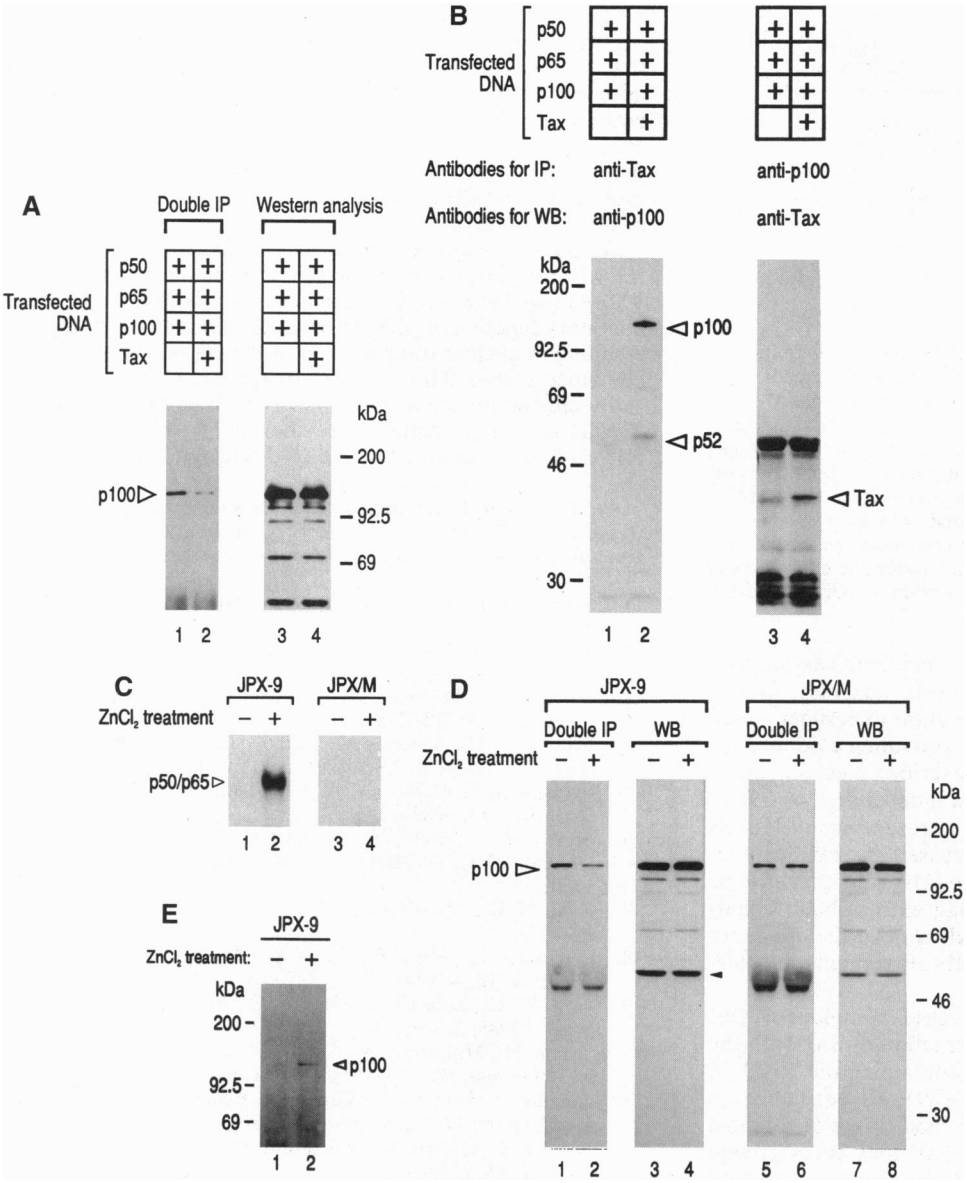

FIG. 3. Effect of Tax on the multimeric NF-κB complexes. (*A* and *B*) Tax associates with p100 and dissociates the multimeric complexes in NTera-2 cells. NTera-2 cells were transfected with expression vectors (each at 2 μg) in combinations as indicated. In *A*, the whole-cell extracts were separated by SDS/PAGE either after the double IP procedure (lanes 1 and 2) or directly (lanes 3 and 4). Western blot analysis was performed with anti-p100 antibody. In *B*, the whole-cell extracts were immunoprecipitated with the indicated antibodies, separated by SDS/PAGE, and examined by Western blot analysis with the antibodies shown. (*C–E*) Tax protein decreases the multimeric NF-κB complexes in JPX-9 Jurkat T cells. JPX-9 and JPX/M cells were treated with $ZnCl_2$ to induce wild-type and mutant Tax proteins, respectively. In *C*, the whole-cell extracts were incubated with a ^{32}P-labeled κB palindromic probe and analyzed by EMSA. The position of the p50–p65 heterodimeric complexes is shown, which was confirmed by a supershift experiment using specific antibodies. In *D*, whole-cell extracts from the untreated or $ZnCl_2$-treated JPX-9 (lanes 1–4) and JPX/M cells (lanes 5–8) were separated by SDS/PAGE either after the double IP procedure (lanes 1, 2, 5, and 6) or directly (lanes 3, 4, 7, and 8). Western blot analysis was performed with anti-p100 antibody. Positions for p100 and p52 (open and solid arrowheads, respectively) are indicated. In *E*, whole-cell extracts from the untreated (lane 1) or the $ZnCl_2$-treated (lane 2) JPX-9 cells were immunoprecipitated with anti-Tax antibody, separated by SDS/PAGE, and examined by Western blot analysis with anti-p100 antibody.

and in this way may relieve inhibition and contribute to nuclear translocation of p50–p65 heterodimers. This mechanism may be unique to Tax, because the multimeric complexes were not dissociated in Jurkat cells during short-term stimulation with phytohemagglutinin, phorbol 12-myristate 13-acetate, or tumor necrosis factor α, which induced significant amounts of NF-κB through degradation of IκB-α (data not shown). Finally, Tax did not increase the processing of p100 into p52, as indicated by the unchanged amounts of both proteins (Fig. 3*D*, lanes 3 and 4; see also Fig. 3*A*, lanes 3 and 4, for NTera-2 cells and ref. 2). This rules out the possibility that Tax might have activated NF-κB by converting p100–p65 into p52–p65 dimers, and it is consistent with the absence of p52/p100-immunoreactive material in the nucleus in experiments shown in Fig. 1.

We also investigated for potential inhibitory effects of p100 on DNA binding of preformed p50–p65 heterodimers *in vitro* in the presence or absence of Tax. We prepared cell lysates containing p50–p65 heterodimers, p100, or Tax by separate transfection of NTera-2 cells with appropriate expression vectors. The lysates were mixed and analyzed by EMSA. When p100 lysates were added, the binding of p50–p65 heterodimers was decreased (Fig. 4, lanes 2, 3, and 6). When Tax lysates were included in addition (otherwise conditions as in lane 3), DNA binding by p50–p65 heterodimers was again restored to uninhibited levels (Fig. 4, lanes 4 and 5). Neither the Tax nor the p100 lysate alone exhibited any binding activity (Fig. 4, lanes 8 and 9), and importantly, Tax did not affect the binding of p50–p65 heterodimers directly (Fig. 4, lane 7). Thus Tax antagonized the inhibition of p50–p65 dimers by p100 *in vitro*.

DISCUSSION

We have shown that HTLV-I Tax can activate NF-κB by targeting cytoplasmic complexes containing p50, p65, and p100. Within such complexes, the C-terminal ankyrin-repeat domain of p100 is required for association with and inhibition of p50–p65 heterodimers, most likely masking their nuclear localization signals. While the precise stoichiometry is not yet known, these p100-containing complexes are clearly different from any considered previously; precursors were thought to sequester p65 or c-Rel in the cytoplasm by forming heterodimers through their dimerization domains located within the RHDs (3–6). Although such heterodimers do form, there is no evidence that Tax targets these heterodimers for nuclear import. Rather, Tax targets the coexisting multimeric complexes; Tax dissociates the multimers and thus allows the previously contained p50–p65 heterodimers to translocate to

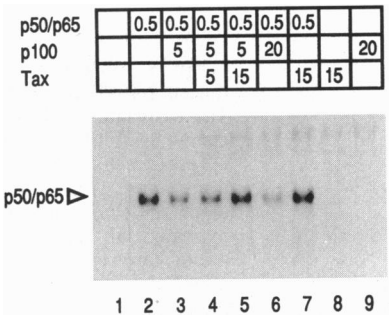

FIG. 4. Tax protein antagonizes the p100-mediated inhibition of DNA binding of p50–p65 heterodimers *in vitro*. NTera-2 cells were transfected with pMT2T-p50 (4 μg)/pMT2T-p65 (4 μg), with pMT2T-p100 (8 μg), with pMT2T-Tax (8 μg), or with pMT2T (8 μg). Whole-cell extracts were prepared and mixed in the amounts (in μg) indicated. The total amount of whole-cell extracts was kept constant by using the extract from NTera-2 cells transfected with pMT2T only. The extract mixtures were incubated with a ^{32}P-labeled κB palindromic probe, and the protein–DNA complexes were analyzed by EMSA. The position of the p50–p65 heterodimeric complexes is shown, which was confirmed by a supershift experiment using specific antibodies.

the nucleus. The multimeric structures and their Tax-induced dissociation were demonstrated by co-IP analyses, first in NTera-2 cells with controlled transfection experiments and then in Tax-inducible Jurkat T cells, providing evidence for the physiological relevance of the described mechanism.

The p100-inhibited complexes exist independent of IκB-α; their normal function may be as a reservoir of NF-κB complexes that are not readily accessed by extracellular signals but allow the signal-responsive IκB-α complexes to be replenished with time. Indeed, strong extracellular signals that lead to near-complete loss of IκB-α nevertheless cause only a fraction of the cytoplasmic NF-κB proteins to translocate to the nucleus (6).

How does Tax dissociate multimeric complexes? Our co-IP data demonstrate a strong interaction of Tax with the N-terminal half of the p100 protein containing the RHD. A mutant of Tax that does not activate NF-κB but that does activate through CRE sites does not bind to the N-terminal half of p100, and another mutant that does transactivate through κB sites but not through CRE sites continues to bind p100 (11). These data suggest that Tax binding to the N-terminal half of p100 can somehow interfere with the inhibitory function of the C-terminal ankyrin domain, as determined in cells and by *in vitro* DNA binding assays (Figs. 1 and 4). The antagonistic effects of Tax on p100 inhibition are not due to increased processing of the precursor (Fig. 3 *A* and *D*). The view that the interaction of Tax with p100 results in NF-κB activation is not incompatible with the proposal (11) that the interaction may serve to establish latency in HTLV-I infections. Cytoplasmic retention of Tax through the interaction with p100 may inhibit nuclear functions of the viral protein, such as the activation of CRE, while activation of NF-κB can proceed in the cytoplasm.

Tax has also been reported to interact with p50, the N-terminal half of p105, and IκB-γ—the C-terminal half of p105 (20, 21). Such interactions were proposed to be responsible for nuclear translocation of otherwise cytoplasmic complexes in transfection experiments (21, 22). However, effects of Tax on IκB-γ may not be physiologically relevant since this inhibitor has not been demonstrated in T cells or in any other human cells. The interaction of Tax with p105, which includes the IκB-γ sequences, or with the ankyrin domain of p100 is much weaker than that with the N-terminal half of p100 (data not shown, and ref. 11). Nonetheless, it is possible that Tax has some affinity for all of these proteins, and interactions with all of them may ultimately contribute to counteract inhibition by ankyrin-domain proteins. This may even include an effect on complexes containing IκB-α, in particular, since Tax expression appeared to cause a more rapid turnover of IκB-α, which may weaken the effectiveness of this inhibitor (2).

We have provided evidence for a cytoplasmic reservoir of NF-κB complexes containing the p100 inhibitory protein; in addition, we have provided evidence that Tax antagonizes the inhibitory function of p100 within such multimeric complexes, resulting in nuclear translocation of the NF-κB heterodimers contained within. This mechanism appears to be physiologically relevant for the activation of NF-κB by HTLV-I Tax in T cells and may contribute to the high levels of activated NF-κB dimers observed in HTLV-I-infected cells.

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