Nuclear Factor κB–dependent Gene Expression Profiling of Hodgkin’s Disease Tumor Cells, Pathogenetic Significance, and Link to Constitutive Signal Transducer and Activator of Transcription 5a Activity

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Abstract
Constitutive nuclear factor (NF)-κB activity is observed in a variety of hematopoietic and solid tumors. Given the distinctive role of constitutive NF-κB for Hodgkin and Reed-Sternberg (HRS) cell viability, we performed molecular profiling in two Hodgkin’s disease (HD) cell lines to identify NF-κB target genes. We recognized 45 genes whose expression in both cell lines was regulated by NF-κB. The NF-κB–dependent gene profile comprises chemokines, cytokines, receptors, apoptotic regulators, intracellular signaling molecules, and transcription factors, the majority of which maintain a marker-like expression in HRS cells. Remarkably, we found 17 novel NF-κB target genes. Using chromatin immunoprecipitation we demonstrate that NF-κB is recruited directly to the promoters of several target genes, including signal transducer and activator of transcription (STAT)5a, interleukin-13, and CC chemokine receptor 7. Intriguingly, NF-κB positively regulates STAT5a expression and signaling pathways in HRS cells, and promotes its persistent activation. In fact, STAT5a overexpression was found in most tumor cells of tested patients with classical HD, indicating a critical role for HD. The gene profile underscores a central role of NF-κB in the pathogenesis of HD and potentially of other tumors with constitutive NF-κB activation.

Key words: oncogene • tumor suppressor • survival • chromatin • microarray

Introduction
Members of the nuclear factor (NF)*-κB transcription factor family regulate immune, inflammatory, and acute phase responses, and their homozygous inactivation in mice results in severe immune system dysfunction (1–3). One of its most important functions is the activation of an antiapoptotic gene expression program (4–6). More recently, NF-κB activation has been connected to cell growth control (7–9). Because signaling pathways that govern proliferation and survival are important for tumor development, NF-κB has an intrinsic oncogenic potential.

Indeed, ample evidence linking Rel/NF-κB activity to oncogenesis has been accumulated in the past years (7–10). Transforming capacity has first been demonstrated for the viral oncprotein v-Rel in vitro and in vivo (10). Moreover, oncogenic viruses, such as human T cell leukemia virus I or Epstein-Barr virus, activate NF-κB as part of the transformation process (11, 12). Similarly, cellular oncoproteins like Her-2/Neu and BCR-ABL induce NF-κB to achieve resistance to apoptosis or enhance transformation capacity (13, 14). Finally, chromosomal rearrangements of genes coding for Rel/NF-κB factors have been observed in
many human hematopoietic and solid tumors, and several human cancer cell types display persistent nuclear NF-κB activity as a result of constitutive activation of IkB kinases (IKKs) or mutations inactivating IkB subunits (7).

Most evidence for the role of Rel/NF-κB in human malignancies came from an analysis of Hodgkin’s disease (HD). HD is the first hematopoietic tumor from which an aberrant constitutive NF-κB activation has been described (15). The malignant mononuclear Hodgkin and multinucleated Reed-Sternberg cells in HD only represent a fraction of the neoplastic lesions that are populated by eosinophils, neutrophils, T cells, B cells, plasma cells, histiocytes, and others. These reactive cells are attracted by cytokines and chemokines abundantly produced by Hodgkin and Reed-Sternberg (HRS) cells (16–18). Molecular single cell analysis has suggested that HRS cells are derived from germinal center B cells or B cells at later differentiation stages (19). Due to the scarcity of malignant cells it has been difficult to define the transforming molecular lesions that lead to the development of HD. Several HRS cell lines have been established and clonal identity with primary HRS cells has been demonstrated for L1236 cells, indicating that HRS cell lines can serve as suitable model systems (20). Constitutively activated NF-κB is a characteristic feature of HRS cell lines and primary cells (15, 21). Interestingly, mutations in the ikbα gene, producing nonfunctional or unstable IkBα proteins, are recurrent molecular abnormalities of HRS cell lines (22–24). However, most primary HRS cells lack mutations in the ikbα gene. Recent data indicate persistently activated IKK complex as the major cause of constitutive NF-κB activity (25). The inhibition of NF-κB by the expression of the superrepressor IkBΔN in various HRS cell lines led to decreased proliferation, enhanced apoptotic response, and strongly impaired tumor growth in immune-deficient mice (21, 26). Constitutive NF-κB activity regulates the expression of genes typically upregulated by HRS cells, including the cell cycle regulatory protein cyclin D2, antiapoptotic proteins Bcl-1/A1, c-IAP2, TNFR-associated factor 1, and Bcl-xL, and the cell surface receptors CD40 and CD86 (26). Thus, an important function of aberrant NF-κB activity in cell growth of malignant cells is well established. However, a determination of the full group of genes controlled by constitutive NF-κB will be important to understand its pathogenetic role in HD and other types of tumors.

For this purpose, we performed large-scale gene expression profiling in L428 and HDLM2 cells. In both cell lines, NF-κB activity could be efficiently blocked by adenosivral expression of the superrepressor IkBΔN, leading to massive spontaneous apoptosis within 48 h after infection. We identified 45 genes, which are affected upon NF-κB inhibition, encoding chemokines, cytokines, receptors, apoptotic regulators, intracellular signaling molecules, and transcription factors. Several known NF-κB target genes were found, which are overexpressed in primary or cultured HRS cells. Besides this, 17 novel genes could be verified as NF-κB targets by Northern blot or RT-PCR analysis. Importantly, most of these genes displayed elevated expression levels in HRS cells. Stimulus- and IKK-dependent induction in non-Hodgkin cells, as well as direct recruitment of NF-κB to promoter regions, confirmed that many of these genes, including signal transducer and activator of transcription (STAT)5a, CCR7, or IL-13 are direct targets of NF-κB. We observed that NF-κB interferes with the Janus kinase/STAT signaling pathway and causes a high level of constitutive STAT5a activity in cultured and primary HRS cells. Because NF-κB controls a complex network of genes with central pathogenic importance in HD, we suggest that NF-κB is a key regulator in this malignancy.

Materials and Methods

Viral Infection. Ad5-IκBΔN and Ad5 control viruses were previously described (26). 10^10–10^12 pfu/ml pelleted cells were resuspended in RPMI 1640 containing 10% FCS at 10^5 cells/ml. Viruses were added (at a multiplicity of infection [m.o.i.] of 300 for L428; m.o.i. of 100 for HDLM2) and cells were incubated for 2 h at 37°C in 5% CO2. After infection, cells were pelleted and resuspended at 3 × 10^5 cells/ml.

Cell Culture. Rhe, Nam, L428, L1236, KMH-2, and HDLM2 cells were grown in RPMI 1640 (GIBCO BRL) supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2 mM l-glutamine, as well as 50 μM β-mercaptopetoehol for 70Z/3 or 1.3E2 cells. Cells were treated with 200 ng/ml PMA (Calbiochem-Novabiochem), 10 μg/ml LPS (Sigma-Aldrich), or 25 μg/ml cycloheximide (CHX; Calbiochem-Novabiochem).

Extracts, Electrophoretic Mobility Shift Assay (EMSA), Western Blotting, and Immunoprecipitation. Preparation of whole cell extracts, Western blotting, and EMSA was performed as previously described (26). STAT5a gel shift oligonucleotides (sc-2565) were purchased from Santa Cruz Biotechnology, Inc. For immunoprecipitation, 400 μg protein whole cell extract was mixed with immunoprecipitation buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, protease and phosphatase inhibitors). Extracts were precleared with protein A–Sepharose for 1 h at 4°C. Immunoprecipitation using 2 μg STAT5a antibody (sc-1656; Santa Cruz Biotechnology, Inc.) was performed for 2 h at 4°C. Samples were washed four times with immunoprecipitation buffer and analyzed by SDS-PAGE and Western blotting. Mouse monoclonal antibodies against phosphotyrosine (05–321; Upstate Biotechnology), rabbit polyclonal antibodies against IkBα (C-21; Santa Cruz Biotechnology, Inc.), STAT5a (66621N; BD Biosciences), or CDK4 (H-22; Santa Cruz Biotechnology, Inc.), as well as horseradish peroxidase–conjugated anti-rabbit or anti–mouse antibodies (New England Biolabs, Inc.) were used for detection.

DNA Microarray Analysis. Total RNA was prepared from Ad5 control- or Ad5-IκBΔN–infected L428 or HDLM2 cells 24 h after infection (RNaseasy Kit; QIAGEN). Samples for Affymetrix microarray analysis were prepared according to the manufacturer’s instructions. The HuGeneFL GeneChip microarray was hybridized with cDNA at 45°C for 16 h, washed, and stained using the GeneChip Fluidics station according to the manufacturer’s instructions. DNA chips were scanned with a GeneChip scanner and signals were processed by the GeneChip expression analysis algorithm (version 3.2; Affymetrix). The quantification of each gene expression was obtained from the hybridization intensities of 20 perfectly matched and mismatched control probe pairs. All chip files were scaled to a uniform intensity value of 1,000. For a comparative chip file, the experimental file (Ad5-IκBΔN–infected cells) was compared with the baseline file (Ad5 control–infected cells).
Genes that fit the following criteria were considered decreased/increased genes upon NF-κB inhibition: the change call was either decreased or induced, the change in the average difference was greater than twofold, the sort score value was >1 or <1, and an absolute call of “presence” was associated with the baseline file.

Northern Blotting. Total RNA preparation and Northern blotting was performed as previously described (26). For the generation of probes, I.M.A.G.E. cDNA clones (Resource Center of the German Human Genome Project, Max-Planck Institute for Molecular Genetics, Berlin, Germany) were either digested or amplified by PCR. Isolated fragments were labeled with Megaprime DNA Labeling System (Amersham Biosciences). Detailed information is available upon request.

RT-PCR. 5 μg total RNA (see above) were reverse transcribed using oligo dT primers and the Superscript™ first strand synthesis RT-PCR system (Life Technologies) according to the manufacturer’s instructions. cDNA was amplified by CombiPol DNA polymerase (Invitrek). To establish relative quantities, serial cDNA dilutions were amplified with β-actin–specific primers (22 cycles) for standardization. To semi-quantify expression levels of potential target genes, cDNAs were amplified with specific PCR primer pairs (30–40 cycles) in a volume of 50 μl. Nucleotide sequences and detailed PCR conditions are available upon request.

Chromatin Immunoprecipitation (ChIP) Assay. ChIP was performed as previously described (27). The following are sequences of promoter–specific primers: IκBα: 5’ GAGGACCCCAATTGAGTGGTGTGCCTGCGCC 3’ (as); c-jun: 5’ CGACTGTAGGAGGGCAGCGG 3’ (as); CD44: 5’ GAGGG-TCACCCCGCCGCCC-G 3’ (as); CCR7: 5’ CCAGAAGGGCG-TGGGCAAGC 3’ (as); STAT5a: 5’ GTGGGCAAGC-GTGGGCAAGC 3’ (as); IL-13: 5’ CCAGAAGGGCGCTCAGAGTCGCTGGTCATAGG 3’ (as); GLUT5: 5’ GTAAGTGTTGCCCGGCTGGG 3’ (as); tissue factor pathway inhibitor (TFPI)-2: 5’ CACAA-ACGCTCCCTCAGGGC 3’ (s), 5’ TCACCCCGCGGCCGCGCCGGCCGGCCG 3’ (as); and GLUT5: 5’ CCAGTATTCCACCATGGAGCC 3’ (s), 5’ GTAAGTGTTGCCCGGCTGGG 3’ (as).

Immunohistochemistry. Paraffin blocks from lymph node biopsies of patients with classical HD were from files of the Consultation and Reference Center for Hematopathology, Free University, Berlin, Germany. The diagnosis of classical HD was according to the criteria of the World Health Organization’s classification (28). Four micrometer sections were deparaffinized and subjected to an antigen retrieval protocol to optimally visualize antigens in paraffin–embedded tissue. For that, tissue sections were immersed in 10 mmol citrate, pH 6.0, and cooked under high pressure in a pressure cooker for 2 min. Sections were incubated with anti-STAT5a antibody (sc-1656; Santa Cruz Biotechnology, Inc.) at a dilution of 1:200. Bound antibody was visualized using the streptavidin–biotin–alkaline phosphatase method and Fast Red as chromogen (kit 5005; Dako). The specificity of STAT5a immunodetection in HRS cells was confirmed by competition with a specific peptide (sc-1656p; Santa Cruz Biotechnology, Inc.).

Results

NF-κB–dependent Gene Profiling in HRS Cells. The identification of target genes is an important step to understand the oncogenic potential of NF-κB and its function in HRS cells. To provide a representative gene profile, we performed a parallel microarray analysis of two different HRS cell lines. We previously established adenovirus–mediated expression of the superrepressor IκBΔN to downmodulate constitutive NF-κB activity in the HRS cell line L428 (26). As a second HRS cell line, HDLM2 cells were used. As observed for L428 cells, the expression of IκBΔN nearly abolished NF-κB DNA binding activity in HDLM2 cells and caused a dramatic growth defect pri-
NF-κB–mediated protection against apoptosis is due to its transcriptional regulation of a distinct set of antiapoptotic genes (26). RNA prepared 24 h after the infection of L428 and HDLM2 cells with either Ad5 control or Ad5-IκBΔN viruses was used for hybridization to high density DNA microarrays. The DNA arrays contained 7,133 gene sequences and expressed sequence tags. Before hybridization, RNA from each of the samples was converted to target according to standard procedures. The hybridized chips were then processed and analyzed as described in Materials and Methods.

A total of 45 genes met the criteria that expression was considered decreased or increased upon NF-κB inhibition in both L428 and HDLM2 cell lines (Fig. 2). Other genes fulfilled the criteria for NF-κB–dependent regulation in only one cell line. In L428 cells, the expression of 15 additional genes was decreased and 6 were increased upon NF-κB inhibition. In HDLM2 cells, 43 additional genes had decreased and 23 had increased expression (Fig. 3). Among the candidates identified as common for both cell lines, the expression of only one gene, l-fucosidase, was increased, indicating a potential repressor function of NF-κB. Expression of all others was decreased greater than twofold, in turn suggesting that these genes are induced by constitutive NF-κB activity in HRS cells.

The target genes could be classified into these groups: cytokines and chemokines, cell surface receptors, cell adhesion molecules, regulators of apoptosis, signaling molecules, transcription factors, and a group of genes with miscellaneous functions (Fig. 2). The presence of several genes known to be NF-κB target in other cell types like lympho-
NF-kB target genes in individual HRS cell lines (A) L428 and (B) HDLM2. Analysis was performed as described in Fig. 2. Genes with decreased expression genes upon NF-kB inhibition are listed first followed by genes with increased expression. Graphical representations of average difference values are shown.

### High Level Expression of NF-kB Target Genes in HRS Cells

All novel candidates identified for both L428 and HDLM2 cells and a group of known NF-kB target genes were selected for Northern analysis to validate NF-kB-dependent expression in HRS cells. Because the microchip data were obtained from two independent experiments and two different cell lines, the rate of false positives should be minimal. For some candidates, semi-quantitative RT-PCR was performed. We analyzed mRNA expression in Ad5-IκBΔN– or Ad5 control–infected L428 and HDLM2 cells, and in uninfected HRS and control cell lines. For all samples we confirmed that NF-kB DNA binding activity was constitutive only in HRS cells and was repressed by IκBΔN (Fig. 4 A). Pronounced NF-kB–dependent regulation could be verified for the vast majority of genes (Fig. 4, B and C). Moreover, the observed change of mRNA expression levels upon NF-kB inhibition correlated well with the DNA microarray data (Fig. 2). CX3CL1, MIP1-α, CCR7, IL-15Rα, CD83, IEX-1, SMAD7, interferon regulatory factor 1, and NF-kB p100, previously proposed to be regulated by NF-kB in other cell types (29, 35–39), could be confirmed as cellular target genes in HRS cells. Abundant CCR7 and CD83 expression was detected exclusively in HRS cells (Fig. 3 B), supporting recent observations (38, 40). Likewise, the mRNA patterns of CX3CL1, IEX-1, IL-15Rα, and p100 indicate strongly elevated expression in HRS cells compared with non-HRS cells (Fig. 4 B).
Among the novel target genes, 17 could be validated by Northern blotting or RT-PCR, namely IL-13, MDC, I-309, EMR1, CD44, ABIN, LSP-1, protein kinase C (PKC)-δ, STAT5a, Spi-B, LPS-induced TNF-α factor (LITAF), HLA-F, glucose transporter protein GLUT5, TPMT, KIAA0084, and RES4–25 (Fig. 4, B and C). The chemokine I-309 was strongly and NF-κB dependently expressed in virally infected cells (Fig. 4 B). In contrast, only weak amounts of mRNA could be detected in noninfected HRS cells, indicating a stimulating event caused by adenoviral infection. The remaining six candidates, like TC21 or NCF2 (Fig. 2), could not be confirmed (unpublished data). This might be caused by the fact that these candidates display very low average values or that the observed change in the average difference was near the cutoff criteria (Fig. 2). In general, the microarray analysis was confirmed by Northern and RT-PCR analysis.

As an important observation, many of the novel target genes, including MDC, IL-13, CD44, ABIN, LSP-1, STAT5a, GLUT5, TPMT, and TFPI-2 display high level expression in HRS compared with non-HRS cells, thus correlating with constitutive NF-κB activity. CD44 and IL-13 have been suggested as crucial factors in the pathogenesis of HD (31, 33).
Novel Target Genes Are Inducible by IKK-dependent Signaling. For additional verification, a subgroup of novel target genes was analyzed in 70Z/3 pre-B lymphoma cells and their IKKy-deficient variant 1.3E2, which is defective in IKK signaling (41). Cells were stimulated with PMA, LPS, or LPS in combination with CHX, and mRNA expression of CCR7, Spi-B, LITAF, PKC-δ, and ABIN was analyzed (Fig. 5). CCR7 and Spi-B mRNA expression was significantly induced by LPS, whereas no or only weak induction was observed with PMA or LPS in the presence of CHX. Because CHX blocks protein synthesis, these observations suggest an additional protein requirement for NF-κB–dependent activation of CCR7 and Spi-B. In contrast, LITAF, PKC-δ, and ABIN mRNAs were induced by all stimuli in 70Z/3 cells. The lack of induction of all five genes in 1.3E2 cells and the results from previous experiments (Fig. 2 and Fig. 4, B and C) reveals that all are regulated through the IKK–NF-κB pathway.

NF-κB Recruitment to Target Promoters. To analyze if NF-κB is recruited to target promoters, ChIP assays (27) were performed with L428 cells. The p65 antibody precipitated aIkBα gene promoter fragment that could be blocked with an antibody-specific peptide, although it did not precipitate the c-Jun promoter. However, both promoter fragments could be precipitated with an anti–c-Jun antibody (Fig. 6 A). These data demonstrate the specificity of the procedure and are in agreement with the conservation of binding sites in the two genes. Next, ChIP assays were performed with L428 cells that were uninfected or infected with Ad5-IκBΔN or Ad5 control. p65 recruitment to the IkBα promoter was strongly diminished in IκBΔN-expressing cells compared with infected and uninfected controls (Fig. 6 B). We also investigated the association of NF-κB with IL-13, CCR7, CD44, STAT5a, TPMT, TFPI-2, or Glut5 promoter regions (Fig. 6 B). In all cases, we observed NF-κB binding in noninfected and Ad5 control–infected cells. Again, no or only weak binding was observed in the presence of peptide or in cells infected with Adv-IκBΔN. Promoter sequences in the databases (GenBank and euGenes) showed that all analyzed genes contain NF-κB binding site motifs according to the consensus sequence GGGRNNYYCC (Fig. 6 B; reference 42). The data provide strong evidence that these genes are under direct transcriptional control of NF-κB.

NF-κB Induces STAT5a Overexpression and Activation in HRS Cells. The transcription factor STAT5a is an interesting novel target gene, because STAT5a activity is linked to cell growth control. Moreover, constitutively activated STAT5a has been observed in a variety of tumors (43, 44). Similar to mRNA expression (Fig. 4 B), we observed high level protein expression of STAT5a in all HRS cell lines but not in non–HRS cells (Fig. 7 A). STAT5a protein expression was dependent on NF-κB because it was reduced in both L428 and HDLM2 cells 48 h after infection with Adv-IκBΔN. Furthermore, a constitutive STAT5a DNA binding activity was observed in the majority of HRS cell lines, most strongly in L1236 and L540 cells (Fig. 7 B). In line with this, constitutive tyrosine phosphorylation, a prerequisite for STAT5a activation, was observed in HRS cells (Fig. 7 C). Intriguingly, NF-κB inhibition led to a rapid loss of STAT5a phosphorylation and DNA binding activity 24 h after infection with Ad5-IκBΔN (Fig. 7, B and C). Thus, NF-κB controls both expression and activation of STAT5a.

Patients with Classical HD Reveal a High Level of STAT5a Expression in Malignant Cells. As predicted from the data obtained with cell lines, all patients tested with classical HD (24 cases) revealed high level cytoplasmic and nuclear staining for STAT5a in >80% of the HRS cells in the lymph node sections (Fig. 7 D and unpublished data). Tonsil sections, as benign tissue, revealed elevated STAT5a expression in germinal center cells, albeit not at the same level as in HRS cells (unpublished data). Nuclear staining of HRS cells implies that STAT5a is constitutively active in primary HRS cells. In contrast to classical HD, only in a subset (4 out of 14 cases) of lymphocyte predominance HD were all malignant lymphocytic and histiocytic cells significantly stained for STAT5a (unpublished data). Overall, STAT5a staining was weaker in lymphocyte predominance HD compared with classical HD.

Discussion

A considerable body of work has linked deregulated NF-κB activity to oncogenesis (7). High level constitutive nuclear NF-κB is a characteristic and important property of the malignant cells of HD (15, 18, 21, 26). There is ample evidence that cell death protection is a key function of constitutive NF-κB activity in HRS cells (Figs. 1 and 2; references 21 and 26). However, additional contributions to the pathogenesis of HD are poorly understood. Our large-scale gene profiling revealed that NF-κB regulates a
Complex network of genes, which are overexpressed in primary and cultured HRS cells (Table I; references 18, 26, 31, 32, 38, 40, and 45–49). A significant fraction of these genes appears to determine important characteristic properties of malignant cells in HD.

In addition to the antiapoptotic function, NF-κB might render tumor cells resistant to chemotherapy, as thiopurine TPMT, which catalyses S-methylation of thiopurines such as 6-mercaptopurine and 6-thioguanine, was identified as a novel target with high level expression in HRS cells (Figs. 2, 4 C, and 6). Indeed, TPMT activity is relevant for chemotherapy treatment, as has been reported for childhood acute lymphoblastic leukemia (50).

The malignant HRS cells invoke the infiltration of reactive cells including granulocytes, plasma cells, and T cells. The expression of the chemokine MDC with the adhesion molecule ICAM-1 and CD86 by HRS cells has been proposed to account for a preferential influx of Th2-type T cells and the suppression of Th1-type immune response (45). Recent data suggested that TNF-α secretion by HRS cells induces eotaxin in fibroblasts of HD tissue, which subsequently recruits T cells and eosinophils (51). Likewise, CX3CL1 and CD83 have the potential to attract T cells and might contribute to T cell influx into the affected lymph nodes (52, 53). Similarly, cytokines like IL-6 and GM-CSF stimulate plasma cells, Th2 cells, and eosinophils (45, 54). Taken together, these NF-κB target genes are strongly implicated to contribute to the architecture of affected lymph nodes in HD.

Tumor cells of classical HD are predominantly found in the interfollicular zone or less frequently in the follicular mantle zone of partially infiltrated nodes (55). Thus, confining tumor cells to distinct lymphoid compartments might be mediated by chemokine receptors like CCR7. In agreement with recent data, CCR7 was determined as a bona fide NF-κB target gene in HRS cells (38). CCR7 might not only contribute to distinct dissemination of neoplastic cells into lymphoid organs, but also seems to have a more general role in tumor cell migration because a critical role in breast cancer metastasis has been described (56).

Likewise, CD44 is expressed at high levels in HRS cells and implicated in the dissemination of HRS cells (31). Moreover, the expression of CD44 splice variant v10 is associated with an unfavorable clinical prognosis (31). Finally, the serine protease inhibitor TFPI-2, which has a proinvasive effect in hepatocellular carcinoma cells, might not only contribute to distinct dissemination of neoplastic cells into lymphoid organs, but also seems to have a more general role in tumor cell migration because a critical role in breast cancer metastasis has been described (56).
also be involved in the migration of HD tumor cells (57). Altogether, NF-κB controls a set of genes that likely regulates tumor cell localization.

Deregulated proliferation is a typical event associated with malignant transformation. Therefore, the overexpression of factors involved in growth control is of great interest. Both IL-13 and CD40 play a critical role in B cell proliferation (18, 58). Remarkably, IL-13–neutralizing antibodies blocked the proliferation of HDLM-2 cells (33). Likewise, the IL-15–IL-15R signaling pathway was suggested as important for tumor propagation in multiple myeloma (59). Our data reveal IL-15R overexpression in HRS cells (Fig. 5). However, a potential role of the IL-15–IL-15R signaling pathway in HD has to be established. Aside from deregulated proliferation, malignant cells display high rates of glucose uptake and glycolysis (60). The overexpression of GLUT5, as observed in this study, might permit the enhanced uptake of fructose and provide a metabolic advantage for HRS cells (61).

As a striking observation, constitutive NF-κB activates the STAT5a signaling pathway both by overexpression and by the induction of tyrosine phosphorylation of STAT5a in cultured HRS cells (Figs. 2, 4, and 7). Notably, all patients analyzed with classical HD express high level activated STAT5a in the entire tumor cell population (Fig. 7 D). These findings establish a new level of complexity in the

Figure 7. NF-κB induces STAT5a overexpression and activation in HRS cells. (A) Whole cell extracts of control and HRS cells, or infected L428 and HDLM2 cells as indicated, were analyzed by Western blotting with anti-STAT5a antibody (top). Loading control blots were probed with anti-CDK4 antibody (bottom). (B) STAT5a DNA binding activity in whole cell lysates of control and HRS or infected L428 and HDLM2 cells was analyzed by EMSA. Specificity of STAT5a–DNA complexes was confirmed in competition experiments (unpublished data). (C) Immunoprecipitations were performed with whole cell lysates of control and HRS or infected L428 and HDLM2 cells. STAT5a tyrosine phosphorylation was detected by Western blotting using antibodies against phosphotyrosine (top left). Analysis of HDLM2 cells is shown in a separate experiment (top right). Blots were reprobed with antibodies against STAT5a (bottom). (D) Patients with classical HD reveal high level of STAT5a expression in malignant cells. Immunohistochemistry of classical HD. All HRS cells (left; arrows indicate representative cells) reveal strong STAT5a staining compared with surrounding benign cells. Specificity of STAT5a detection was confirmed by competition with a specific peptide (right).
oncogenic function of NF-κB. STAT5a has been implicated in hematopoietic cell growth and tumor development and may therefore present an important downstream effector of NF-κB (43, 44). Because STAT5a regulates cell cycle progression via activating D-type cyclins (62) and inhibits apoptosis by stimulating Bcl-xL expression (63), it might contribute to cyclin D2 and Bcl-xL induction in HRS cells in synergism with NF-κB (26). Because cyclin D2 could not be detected as a direct NF-κB target gene in the microarray analysis 24 h after Adv-lkBΔN infection but is affected at later time points (26), NF-κB could regulate cyclin D2 expression in part via STAT5a. In fact, both cyclin D2 and Bcl-xL contain functional STAT5 binding sites in their promoter regions (64, 65).

Aside from HD, the expression of aberrant NF-κB activity or mutant rel/nfkb genes has been noted in many human hematopoietic (e.g., multiple myeloma, adult T cell leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, and B cell leukemia and lymphoma) and solid tumors (e.g., head and neck squamous cell HNSCC], breast, colon, and ovarian carcinoma; reference 7). Correspondingly, many of the NF-κB target genes are overexpressed in these tumors and are implicated to contribute to their pathogenesis (Table I). In particular GLUT5, Bcl-xL,

| Gene | Known target | Verified target | Expression in HRS cells^a | Expression in other tumors | Potential involvement in pathogenesis |
|------|--------------|----------------|----------------------------|-----------------------------|-------------------------------------|
| IL-6 | +            | +              | +                          | Multiple myeloma, HNSCC     | Induction of plasma cell infiltration |
| GM-CSF | +           | +              | +                          | HNSCC                       | Regulates eosinophil proliferation   |
| CX3CL1 | +           | +              | +                          | Multiple myeloma            | Recruits T cells to lymphoma         |
| ICAM-1 | +           | +              | +                          | Recruits T cells to lymphoma |
| CD83 | +            | +              | +                          | Recruits T cells to lymphoma |
| MDC | +            | +              | +                          | Recruits Th2-type T cells to lymphoma |
| CD86 | +            | +              | +                          | B cell leukemia             |
| Lymphotxin-α | +         | +              | —                          | Interacts with T cells, induces anergy? |
| TNF-α | +            | +              | +                          | Inflammatory mediator       |
| IL-13 | +            | +              | +                          | Multiple myeloma, ATL       | Proliferation, survival              |
| IL-15Ra | +           | +              | +                          | Multiple myeloma, ATL       | Proliferation, survival              |
| CD40 | +            | +              | +                          | B cell lymphoma, carcinoma  | Proliferation, survival              |
| STAT5a | +            | +              | +                          | ATL, CML, ALL               | Proliferation, survival              |
| GLUT5 | +            | +              | +                          | Breast carcinoma            | Metabolic advantage                 |
| IEX-1 | +            | +              | +                          | —                           | Antia apoptotic                      |
| Bcl-xL | +            | +              | +                          | Breast carcinoma            | Antia apoptotic                      |
| A1/Bfl-1 | +          | +              | +                          | Gastric and colon carcinoma | Antia apoptotic                      |
| c-IAP2 | +            | +              | +                          | —                           | Antia apoptotic                      |
| TRAF1 | +            | +              | +                          | —                           | Antia apoptotic                      |
| TPMT | +            | +              | +                          | ALL                         | S-methylaon of thiopurines           |
| CCR7 | +            | +              | +                          | ATL, breast carcinoma       | Dissemination into lymphoid organs   |
| TFPI-2 | +            | +              | +                          | Ovarian carcinoma, HCC      | Invasion                             |
| CD44 | +            | +              | +                          | Colon and breast carcinoma, B-CLL, multiple myeloma | Dissemination in lymphoid organs associated with high risk of relapse |
| ABIN | +            | +              | +                          | —                           | ?                                    |
| LSP-1 | +            | +              | +                          | B cell leukemia and lymphoma | ?                                    |
| NF-κB2/p100 | +       | +              | +                          | Breast and colon carcinoma | ?                                    |

Known target, NF-κB regulation was previously described (26, 29, 38, 77) for HRS or non-HRS cells; Verified target, NF-κB regulation in HRS cells was determined by Northern/RT-PCR; ATL, adult T cell leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; HCC, hepatocellular carcinoma cell; B-CLL, B cell chronic lymphocytic leukemia.

^a Genes for which the high level expression in primary or cultured HRS cells was described in the literature (18, 26, 31, 32, 38, 40, 45–49).

^b Genes for which the high level expression in primary or cultured HRS cells was demonstrated in this study.
CCR7, CD44, and p100 are overexpressed in breast carcinoma (7, 56, 61, 66, 67). In colon carcinoma, high levels of CD44, Bcl-1/A1, and p100 have been observed (7, 67, 68). A high concentration of cytokines IL-6 and GM-CSF are produced by tumor cells of patients with HNSCC (69), and Erika Scharschmidt, Karin Ganzel, and Signe Knespel for excellent technical assistance.

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In summary, this study underlines a fundamental importance of NF-κB in HD. NF-κB controls a complex network of genes, which promotes the specific architecture of Hodgkin lymphoma, supports proliferation and migration, and confers resistance to apoptosis. Pharmacological manipulation of the NF-κB system or of selected target genes might have a therapeutic potential for HD and other neoplastic malignancies.

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References

1. Baueuerle, P.A., and D. Baltimore. 1996. NF-κB: ten years after. Cell. 87:13–20.
2. Baldwin, A.S., Jr. 1996. The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. 14:649–683.
3. Gerondakis, S., M. Grossmann, Y. Nakamura, T. Pohl, and R. Grumont. 1999. Genetic approaches in mice to understand Rel/NF-κB and IκB function: transgenics and knockouts. Oncogene. 18:6888–6895.
4. Sonenshein, G.E. 1997. Rel/NF-κB transcription factors and the control of apoptosis. Semin. Cancer Biol. 8:113–119.
5. Ghosh, S., M.J. May, and E.B. Kopp. 1998. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16:225–260.
6. Barkett, M., and T.D. Gilmore. 1999. Control of apoptosis by Rel/NF-κB transcription factors. Oncogene. 18:6910–6924.
7. Rayet, B., and C. Gelinas. 1999. Aberrant rel/nfkb genes and activity in human cancer. Oncogene. 18:6938–6947.
8. Baldwin, A.S. 2001. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. J. Clin. Invest. 107:241–246.
9. Chen, F., V. Castranova, and X. Shi. 2001. New insights into the role of nuclear factor-κB in cell growth regulation. Am. J. Pathol. 159:387–397.
10. Gilmore, T.D. 1999. Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. Oncogene. 18:6925–6937.
11. Cahir McFarland, E.D., K.M. Izumi, and G. Mosialos. 1999. Epstein-barr virus transformation: involvement of latent membrane protein 1-mediated activation of NF-κB. Oncogene. 18:6959–6964.
12. Sun, S.C., and D.W. Ballard. 1999. Persistent activation of NF-κB by the tax transforming protein of HTLV-1: hijacking cellular IκB kinases. Oncogene. 18:6948–6958.
13. Reuther, J.Y., G.W. Reuther, D. Cortez, A.M. Pendergast, and A.S. Baldwin, Jr. 1998. A requirement for NF-κB activation in Bcr-Abl-mediated transformation. Genes Dev. 12: 968–981.
14. Zhou, B.P., M.C. Hu, S.A. Miller, Z. Yu, W. Xia, S.Y. Lin, and M.C. Hung. 2000. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-κB pathway. J. Biol. Chem. 275:8027–8031.
15. Bargou, R.C., C. Leng, D. Krappmann, F. Emmerich, M.Y. Mapara, K. Bonmert, H.D. Royer, C. Scheidereit, and B. Dörken. 1996. High-level nuclear NF-κB and Oct-2 is a common feature of cultured Hodgkin/Reed-Sternberg cells. Blood. 87:4340–4347.
16. Hsu, S.M., and P.L. Hsu. 1994. The nature of Reed-Sternberg cells: phenotype, genotype, and other properties. Crit. Rev. Oncog. 5:213–245.
17. Kaufman, D., and D.L. Longo. 1992. Hodgkin’s disease. Crit. Rev. Oncol. Hematol. 13:135–187.
18. Staudt, L.M. 2000. The molecular and cellular origins of Hodgkin’s disease. J. Exp. Med. 191:207–212.
19. Marafioti, T., M. Hummel, I. Anagnostopoulou, H.D. Foss, D. Huhn, and H. Stein. 1999. Classical Hodgkin’s disease and follicular lymphoma originating from the same germinal cen-
ter B cell. J. Clin. Oncol. 17:3804–3809.
20. Kanzler, H., M.L. Hansmann, U. Kapp, J. Wolf, V. Diehl, K. Rajewsky, and R. Kuppers. 1996. Molecular single cell analysis demonstrates the derivation of a peripheral blood-derived cell line (L1236) from the Hodgkin/Reed-Sternberg cells of a Hodgkin’s lymphoma patient. Blood. 87:3429–3436.
21. Bargou, R.C., F. Emmerich, D. Krappmann, K. Bommert, M.Y. Mapara, W. Arnold, H.D. Royer, E. Grinstein, A. Greiner, C. Scheideret, et al. 1997. Constitutive nuclear factor-κB-RelA activation is required for proliferation and survival of Hodgkin’s disease tumor cells. J. Clin. Invest. 100:2961–2969.
22. Emmerich, F., M. Meiser, M. Hummel, G. Demel, H.D. Foss, F. Jundt, S. Mathas, D. Krappmann, C. Scheideret, H. Stein, et al. 1999. Overexpression of IκBα without inhibition of NF-κB activity and mutations in the IκBα gene in Reed-Sternberg cells. Blood. 94:3129–3134.
23. Cabannes, E., G. Khan, F. Aillet, R.F. Jarrett, and R.T. Hay. 1999. Mutations in the IκBα gene in Hodgkin’s disease suggest a tumour suppressor role for IκBα. Oncogene. 18:3063–3070.
24. Jungnickel, B., A. Staratschek-Jox, A. Brauninger, T. Spieker, J. Wolf, V. Diehl, M.L. Hansmann, K. Rajewsky, and R. Kuppers. 2000. Clonal deleterious mutations in the IκBα gene in the malignant cells in Hodgkin’s lymphoma. J. Exp. Med. 191:395–402.
25. Krappmann, D., F. Emmerich, U. Kordes, E. Scharschmidt, B. Dorken, and C. Scheideret. 1999. Molecular mechanisms of constitutive NF-κB/Rel activation in Hodgkin-Reed-Sternberg cells. Oncogene. 18:943–953.
26. Hinz, M., P. Lober, S. Mathas, D. Krappmann, B. Dorken, and C. Scheideret. 2001. Constitutive NF-κB maintains high expression of a characteristic gene network, including CD40, CD86, and a set of antiapoptotic genes in Hodgkin/Reed-Sternberg cells. Blood. 97:2798–2807.
27. Saccani, S., P. Fantano, and G. Natoli. 2001. Two waves of nuclear factor κB recruitment to target promoters. J. Exp. Med. 193:1351–1359.
28. Jaffe, E.S., N.L. Harris, H. Stein, and J.W. Vardiman. 2001. World Health Organization Classification of Tumors: Pathology and Genetics of Hematopoietic and Lymphoid Tissues. IARC Press, Lyon. 352 pp.
29. Pahl, H.L. 1999. Activators and target genes of Rel/NF-κB transcription factors. Oncogene. 18:6853–6866.
30. Xerri, L., N. Carucci, P. Parc, J. Hassoun, and F. Birg. 1995. Frequent expression of FAS/APO-1 in Hodgkin’s disease and anaplastic large cell lymphomas. Histopathology. 27:235–241.
31. Beham-Schmid, C., K.H. Heider, G. Hoeftler, and K. Zatloukal. 1998. Expression of CD44 splice variant v10 in Hodgkin’s disease is associated with aggressive behaviour and high risk of relapse. J. Pathol. 186:383–389.
32. Pullford, K., M. Jones, A.H. Banham, E. Haralambeva, and D.Y. Mason. 1999. Lymphocyte-specific protein 1: a specific marker of human leucocytes. Immunology. 96:262–271.
33. Kapp, U., W.C. Yeh, B. Patterson, A.J. Elia, D. Kagi, A. Ho, A. Hessel, M. Tipsword, A. Williams, C. Mirtsos, et al. 1999. Interleukin 13 is secreted by and stimulates the growth of Hodgkin and Reed-Sternberg cells. J. Exp. Med. 189:1939–1946.
34. Skinnider, B.F., A.J. Elia, R.D. Gascoyne, L.H. Trumper, F. von Bonin, U. Kapp, B. Patterson, B.E. Snow, and T.W. Mak. 2001. Interleukin 13 and interleukin 13 receptor are frequently expressed by Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma. Blood. 97:250–255.
35. Garcia, G.E., Y. Xia, S. Chen, Y. Wang, R.D. Ye, J.K. Harrison, K.B. Bacon, H.G. Zerwas, and L. Feng. 2000. NF-κB-dependent fractalkine induction in rat aortic endothelial cells stimulated by IL-1β, TNFα, and LPS. J. Leukoc. Biol. 67:577–584.
36. Ardesha, K.M., A.R. Pizzey, S. Devereux, and A. Khwaja. 2000. The PI3 kinase, p38 SAP kinase, and NF-κB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. Blood. 96:1039–1046.
37. Mariner, J.M., V. Lantz, T.A. Waldmann, and N. Azimi. 2001. Human T cell lymphotropic virus type I Tax activates IL-15Rα gene expression through an NF-κB site. J. Immunol. 166:2602–2609.
38. Hopken, U.E., J.H. Foss, D. Meyer, M. Hinz, K. Leder, H. Stein, and M. Lipp. 2002. Up-regulation of the chemokine receptor CCR7 in classical but not in lymphocyte predominant Hodgkin disease correlates with distinct dissemination of neoplastic cells in lymphoid organs. Blood. 99:1109–1116.
39. Bitzer, M., G. von Gersdorff, D. Liang, A. Dominguez-Rosales, A.A. Beg, M. Rojkind, and E.P. Bottinger. 2000. A mechanism of suppression of TGFβ/SMAD signaling by NF-κB/RelA. Genes Dev. 14:187–197.
40. Sorg, U.R., T.M. Morse, W.N. Patton, B.D. Hock, H.B. Angus, B.A. Robinson, B.M. Coils, and D.N. Hart. 1997. Hodgkin’s cells express CD83, a dendritic cell lineage associated antigen. Pathology. 29:294–299.
41. Yamaoka, S., G. Courtis, C. Besia, S.T. Whiteside, R. Weil, F. Agou, H.E. Kirk, R.J. Kay, and A. Israel. 1998. Complementation cloning of NEMO, a component of the IκB kinase complex essential for NF-κB activation. Cell. 93:1231–1240.
42. Baeuerle, P.A. 1991. The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochim. Biophys. Acta. 1072:63–80.
43. Levy, D.E., and D.G. Gilliland. 2000. Divergent roles of STAT1 and STAT5 in malignancy as revealed by gene disruptions in mice. Oncogene. 19:2505–2510.
44. Lin, T.S., S. Mahajan, and D.A. Frank. 2000. STAT signaling in the pathogenesis and treatment of leukemias. Oncogene. 19:2496–2504.
45. Poppema, S., and A. van den Berg. 2000. Interaction between host T cells and Reed-Sternberg cells in Hodgkin lymphomas. Semin. Cancer Biol. 10:345–350.
46. Schlafer, D., M. March, S. Krajewski, G. Laurent, J. Pris, G. Delol, J.C. Reed, and P. Brouset. 1995. High expression of the bcl-x gene in Reed-Sternberg cells of Hodgkin’s disease. Blood. 85:2671–2674.
47. Messineo, C., M.H. Jamerson, E. Hunter, R. Braziel, A. Bagg, S.G. Irving, and J. Cosman. 1998. Gene expression by single Reed-Sternberg cells: pathways of apoptosis and activation. Blood. 91:2443–2451.
48. Durkop, H., H.D. Foss, G. Demel, H. Klotzbach, C. Hahn, and H. Stein. 1999. Tumor necrosis factor receptor-associated factor 1 is overexpressed in Reed-Sternberg cells of Hodgkin’s disease and Epstein-Barr virus-transformed lymphoid cells. Blood. 93:617–623.
49. Van Gool, S.W., J. Delabie, P. Vandenberghhe, L. Coorevits, C. De Wolf-Peeters, and J.L. Ceuppens. 1997. Expression of B7-2 (CD86) molecules by Reed-Sternberg cells of Hodgkin’s disease. Leukemia. 11:846–851.
50. McLeod, H.L., E.Y. Krynetski, M.V. Relling, and W.E. Evans. 2000. Genetic polymorphism of thiopurine methyltransferase and its clinical relevance for childhood acute lymphoblastic leukemia. *Leukemia*. 14:567–572.

51. Jundt, F., I. Anagnostopoulos, K. Bommert, F. Emmerich, G. Muller, H.D. Foss, H.D. Royer, H. Stein, and B. Dorken. 1999. Hodgkin/Reed-Sternberg cells induce fibroblasts to secrete eotaxin, a potent chemotactant for T cells and eosinophils. *Blood*. 94:2065–2071.

52. Kanazawa, N., T. Nakamura, K. Tashiro, M. Muramatsu, K. Morita, K. Yoneda, K. Inaba, S. Imamura, and T. Honjo. 1999. Fractalkine and macrophage-derived chemokine: T cell-attracting chemokines expressed in T cell area dendritic cells. *Eur. J. Immunol*. 29:1925–1932.

53. Scholler, N., M. Hayden-Ledbetter, K.E. Hellstrom, I. Hellstrom, and J.A. Ledbetter. 2001. CD83 is a sialic acid-binding Ig-like lectin (Siglec) adhesion receptor that binds monocytes and a subset of activated CD8⁺ T cells. *J. Immunol*. 166: 3865–3872.

54. Burgess, A.W., and D. Metcalf. 1980. The nature and action of granulocyte-macrophage colony stimulating factors. *Blood*. 56:947–958.

55. Stein, H., M. Hummel, H. Dürkop, H.D. Foss, and H. Herbst. 1998. Biology of Hodgkin’s disease. In *The Lymphomas*. T.A.L. Canellos and J. Skalar, editors. WB Saunders Co., Philadelphia. 287 pp.

56. Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, et al. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 410:50–56.

57. Neaud, V., T. Hisaka, A. Monvoisin, C. Bedin, C. Balabaud, D.C. Foster, A. Desmouliere, W. Kisiel, and J. Rosenbaum. 2000. Paradoxical pro-invasive effect of the serine proteinase inhibitor tissue factor pathway inhibitor-2 on human hepatocellular carcinoma cells. *Anticancer Res.* 20:4619–4622.

58. Chen, Z., P.S. Malhotra, G.R. Thomas, F.G. Ondrey, D.C. Delgado-Lopez, J. Baselga, F. Nualart, and J.C. Vera. 1996. Expression of functional interleukin-15 receptor and cell-mediated immunity. *Annu. Rev. Immunol.* 16:111–135.

59. Grewal, I.S., and R.A. Flavell. 1998. CD40 and CD154 in the heterogeneous expression of NF-κB by PKA stimulates transcriptional activity by NF-κB targets at the pre-B to immature B cell transition. *J. Biol. Chem.* 273:35565–35569.

60. Dang, C.V., and G.L. Semenza. 1999. Oncogenic alterations of metabolism. *Trends Biochem. Sci.* 24:68–72.

61. Badur, I., I. Marschitz, T. Henn, A. Egle, and R. Greil. 2000. Expression of functional interleukin-15 receptor and autocrine production of interleukin-15 as mechanisms of tumor propagation in multiple myeloma. *Blood*. 95:610–618.

62. Niesvizky, R., D. Siegel, and J. Michaeli. 1993. Biology and treatment of multiple myeloma. *Blood Rev.* 7:24–33.

63. Cook, G., M. Dumbar, and I.M. Franklin. 1997. The role of adhesion molecules in multiple myeloma. *Acta Haematol.* 97: 81–89.

64. Stein, B., A.S. Baldwin, Jr., D.W. Ballard, W.C. Greene, P. Angel, and P. Herrlich. 1993. Cross-coupling of the NF-κB p65 and Fox/Jun transcription factors produces potentiated biological function. *EMBO J.* 12:3879–3891.

65. Zhong, H., R.E. Voll, and S. Ghosh. 1998. Phosphorylation of NF-κB p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol. Cell.* 1:661–671.