The NPM-ALK oncoprotein abrogates CD30 signaling and constitutive NF-κB activation in anaplastic large cell lymphoma

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Summary

NPM-ALK characterizes anaplastic large cell lymphoma (ALCL), as does the high expression of CD30, a feature shared with H-RS cells of classic Hodgkin's lymphoma. In H-RS cells, ligand-independent signaling by overexpressed CD30 drives constitutive NF-κB activation, which is absent in ALCL cells. Here we show that NPM-ALK impedes CD30 signaling and NF-κB activation, dependent on both ALK kinase activity and the N-terminal NPM domain. NPM-ALK transduction into H-RS cell lines abrogates recruitment and aggregation of TRAF proteins, inducing an ALCL-like morphology and phenotype. TRAF2 associates with NPM-ALK at a consensus binding motif located in the kinase domain. Thus, NPM-ALK abrogates CD30-driven NF-κB activation and can also induce an ALCL phenotype, distinguishing ALCL cells from H-RS cells of T cell origin.

Introduction

Anaplastic large cell lymphoma (ALCL) is a group of large-cell neoplasms of T lymphocytes characterized by subtotal effacement of the lymph node structure, prominent sinusoidal diffusion with spread into adjacent paracortical areas, and bizarre/anaplastic morphology (Kadin, 1994). ALCL cells are characterized by expression of EMA, clusterin, and usual absence of CD15, discriminating them from Hodgkin/Reed-Sternberg (H-RS) cells (Jaffe, 2001). NPM-ALK has been identified in ALCL cells as a chimeric protein generated by a chromosomal translocation t(2;5)(p23;q35) that fuses nucleophosmin (NPM) sequences on chromosome 5 to anaplastic lymphoma kinase (ALK) sequences on chromosome 2 (Morris et al., 1994).

NPM is a ubiquitously expressed nuclear protein responsible for protein shuttling between the cytoplasm and nucleus (Borer et al., 1989). NPM has been proposed to function in ribosomal protein assembly and transport (Olson et al., 1986), and also as a molecular chaperone that prevents proteins from aggregating in the crowded environment of the nucleolus (Szebeni and Olson, 1999).

ALK is a receptor tyrosine kinase, the expression of which is normally restricted to neural tissues (Iwahara et al., 1997). When NPM-ALK forms a hexamer at the NPM domain, the oligomerized ALK domain acts as a constitutively active kinase which is a property of the NPM-ALK oncoprotein (Bai et al., 1998; Bischof et al., 1997). NPM-ALK is capable of transforming fibroblasts (Wellmann et al., 1997), and inducing a T cell lymphoma and plasma cell tumors in mice (Chiarle et al., 2003).

The presence of NPM-ALK chimeric protein is characteristic of most systemic/nodal ALCL, and these lymphomas represent a clinicopathological entity (Jaffe, 2001).

ALCL cells are characterized by a high level of CD30 expression that is a phenotype shared with H-RS cells of classic Hodgkin's lymphoma (HL) (Jaffe, 2001; Kadin, 1994; Stein et al., 2000). CD30 is a member of the TNFR superfamily (Chiarle et al., 1999; Horie and Watanabe, 1998), and high expression of CD30 in H-RS cells results in ligand-independent signaling of CD30 that drives constitutive activation of NF-κB (Horie et al., 2002b, 2003). However, NF-κB activation is not observed in ALCL cells in the presence of equivalent levels of CD30 expression (Bargou et al., 1996). Furthermore, inability to activate NF-κB in ALCL cell lines results in differences in biological responses to CD30 activation (Mir et al., 2000). Since aberrant

SIGNIFICANCE

The chimeric oncoprotein NPM-ALK has transforming capacity through its tyrosine kinase activity. This study provides evidence for a novel function of this chimeric tyrosine kinase oncoprotein, presumably based on the chaperone activity of the partner domain, an insight into the function of chimeric tyrosine kinases in lymphomagenesis. Furthermore, NPM-ALK induction of an ALCL-like phenotype in H-RS cells of T lymphocyte origin provides insight into the pathology of HL and ALCL, and the foundation for a conceptual classification of lymphomas with high CD30 expression.

expression of various cytokines in HL cells is considered to be dependent mainly on NF-κB activation, presence or absence of NF-κB activation in HL and ALCL cells may explain differences in clinical symptoms and lymph node histology between these lymphomas (Leoncini et al., 1990).

Tumor necrosis factor receptor (TNFR)-associated factor (TRAF) proteins are adaptor molecules that associate with the cytoplasmic region of TNFR superfamily members, and link these receptors with downstream kinase cascades. Thus far, six TRAF proteins, TRAF1 to TRAF6, have been identified, of which TRAF4 is not known to interact with any receptors (Inoue et al., 2000; Wajant et al., 2001). Ligation of TNF superfamily members with their cognate receptors leads to recruitment of a defined set of TRAF proteins to the receptors, which results in activation of transcription factors, nuclear factor κB (NF-κB), and activator protein-1 (AP-1) (Baud et al., 1999; Malinin et al., 1997; Nishitoh et al., 1998). The cytoplasmic tail of CD30 interacts with TRAF 1, 2, 3, and 5, among which TRAF2 and TRAF5 can mediate signals that activate NF-κB (Horie and Watanabe, 1998; Schneider and Hubinger, 2002). Transcription factors activated by TRAF signaling can induce expression of target genes involved in various aspects of cellular and immune functions. In addition, activation of NF-κB and AP-1 has been shown to protect cells from apoptosis via transcription of anti-apoptotic genes (Beg and Baltimore, 1996; Minden and Karin, 1997). Thus, TRAF-mediated signals play important roles in regulating cell survival, proliferation, and stress responses.

In previous studies, we demonstrated that ligand-independent signaling by overexpressed CD30 is abrogated by transduction of a decoy CD30 lacking the cytoplasmic region, which causes downregulation of IL-13 and induces apoptosis of H-RS cells (Horie et al., 2002b). We further demonstrated that cytoplasmic aggregation of TRAF proteins and colocalization of NIK, IKK, and IκBα with TRAF aggregates mediates continuous signaling by the overexpressed CD30 (Horie et al., 2002a).

Constitutive activation of NF-κB appears to be a basis for the characteristic clinical features of HL and proliferation of H-RS cells. Absence of NF-κB activation in the presence of comparable levels of CD30 expression in ALCL cells suggests that expression of the chimeric oncoprotein NPM-ALK may explain different consequences of CD30 signaling in HL and ALCL. To study the molecular mechanisms that discriminate between ALCL and H-RS cells, we here characterize the function of NPM-ALK in terms of the CD30-TRAF-NF-κB signaling pathway. The results reveal a blockade of CD30 signaling by NPM-ALK, which depends on both ALK kinase activity and presence of the N-terminal NPM domain. It is further shown that wild-type NPM can be phosphorylated by NPM-ALK. Association of TRAF2 with CD30 is inhibited by wild-type NPM in the presence of NPM-ALK in a dose-dependent manner. Moreover, transduction of NPM-ALK into H-RS cell lines of T lymphocyte origin induces morphological and phenotypic changes characteristic of ALCL cells. These results reveal a unique function of the chimeric NPM-ALK tyrosine kinase oncoprotein and indicate that NPM-ALK modulates the phenotype of CD30-expressing tumor cells.

Results

Lack of NF-κB activation and TRAF protein aggregation in ALCL cells

High expression of CD30 triggers ligand-independent signaling in H-RS cells, resulting in constitutive activation of NF-κB (Horie et al., 2002a, 2002b, 2003). However, in ALCL cells, no NF-κB activation is observed in the presence of high levels of CD30 expression (Bargou et al., 1996). These data suggest differences in CD30 signaling between ALCL cells and H-RS cells. To understand the mechanisms for these differences, we first confirmed the expression levels of CD30 and the status of NF-κB activation in cell lines derived from ALCL and HL. Northern blot and immunoblot analyses did not show any appreciable differences in the levels of CD30 expression among cell lines derived from ALCL and H-RS cells. An HEK293 transformant (293CD30) showed a similar level of CD30 expression compared with H-RS cell lines (Figures 1Aa–1Ac). NF-κB activation in H-RS cell lines and 293CD30 cells was clearly demonstrated by electrophoretic mobility shift assay (EMSA), but not in ALCL cell lines that express NPM-ALK chimeric oncoprotein (Figures 1Ad and 1Ae).

Since we previously demonstrated CD30 signal-dependent cytoplasmic aggregation of TRAF proteins in H-RS cells (Horie et al., 2002a, 2002b), we next examined aggregation of TRAF proteins in ALCL cell lines by laser confocal immunofluorescence microscopy. In all three ALCL cell lines studied, no aggregation of TRAF2 and TRAF5 was observed, showing diffuse distribution in the cytoplasm, whereas in a H-RS cell line, L540,

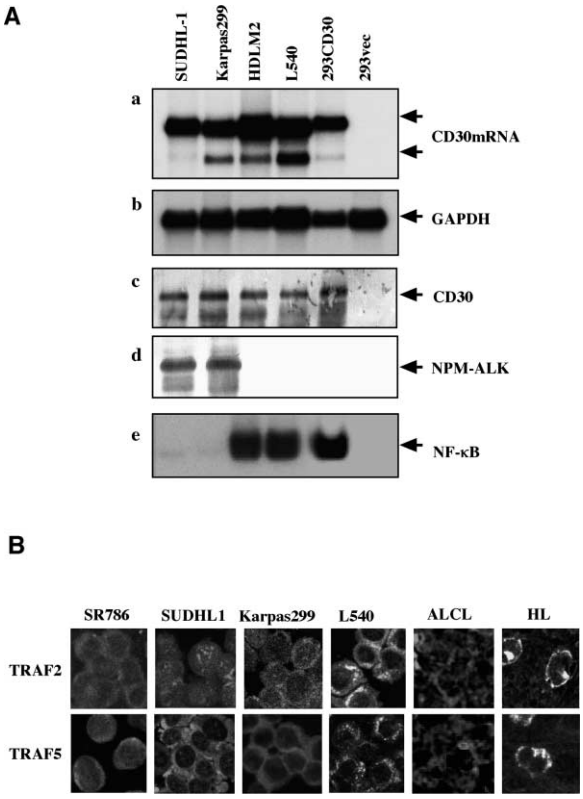

Figure 1. Absence of NF-κB activation and TRAF protein aggregation in ALCL cells

A: NF-κB is not activated in ALCL cell lines in the presence of high levels of CD30 expression. **a and b:** Northern blot analysis of CD30 (**a**) and GAPDH transcripts (**b**). **c and d:** immunoblot analysis of CD30 protein (**c**) and NPM-ALK (**d**). **e:** EMSA with NF-κB probe.

B: Analysis by confocal microscopy of TRAF proteins in ALCL cells. SR786, SUDHL-1 and Karpas 299 are ALCL cell lines, whereas L540 is an H-RS cell line of T cell origin. Right two columns show representative results of biopsied lymph nodes of ALCL and Hodgkin's lymphoma (HL).

aggregation of TRAF proteins was evident as previously reported (Figure 1B). Immunoblot analysis showed no differences in the expression levels of TRAF proteins among cell lines derived from ALCL and H-RS cells (data not shown). Then, we studied distribution of TRAF proteins in biopsied lymph nodes (LN) of ALCL patients. Five LN samples of ALCL and the same number of LN of HL as controls were included. No aggregation of TRAF proteins was observed in ALCL cells, whereas TRAF aggregation was clearly demonstrated in H-RS cells in the LN of HL. Representative results are shown in Figure 1B. These in vitro and in vivo observations suggest that CD30-NF-κB signaling pathway is interrupted downstream of CD30 and upstream of TRAF proteins in ALCL cells. These data also suggest that CD30 signaling may be affected by the presence of the chimeric oncoprotein NPM-ALK (Figure 1Ae).

NPM-ALK interrupts association of TRAF2 with the cytoplasmic tail of CD30

We previously showed that in H-RS cells, highly expressed CD30 recruits TRAF proteins to the cytoplasmic tail, leading to ligand-independent signaling (Horie et al., 2002b). Since CD30-overexpressing ALCL cells do not show NF-κB activation, we next tested the possibility that NPM-ALK may interfere with the association between TRAF proteins and the cytoplasmic tail of CD30. In transiently transfected HEK293 cells, coimmunoprecipitation of CD30 and TRAF2 was clearly detected as reported previously. However, when NPM-ALK was transduced simultaneously with these proteins, the amount of coimmunoprecipitated TRAF2 was greatly diminished, although not totally abolished (Figure 2A, left upper panel). Thus, we next studied association of endogenous TRAF proteins with CD30 in ALCL cell lines. No coimmunoprecipitation was observed in these cells, whereas it was clearly demonstrated in H-RS cell lines (Figure 2A, right upper panel). These results, as well as the absence of cytoplasmic aggregation of TRAF proteins in ALCL cells as demonstrated above, suggest that NPM-ALK interferes with the interaction between CD30 and TRAF proteins, and also between TRAF proteins.

Next we tested NF-κB activation by transient reporter gene assays using a luciferase construct with an NF-κB driven promoter. Transduction of NPM-ALK inhibited NF-κB activation induced by CD30 overexpression. However, this inhibition was not observed when a kinase-negative mutant of NPM-ALK was coexpressed (Figure 2B, left panel). Transduction of the wild-type NPM-ALK, but not a kinase-negative mutant, inhibited NF-κB activation in L540 and 293CD30 cell lines where NF-κB is constitutively activated (Figure 2B, right panel). Thus, NPM-ALK appears to inhibit the CD30-NF-κB signaling pathway as a result of its kinase activity.

We next examined whether recruitment and aggregation of TRAF proteins are inhibited by NPM-ALK. Distribution of TRAF proteins in L540 and 293CD30 cells was studied by laser confocal immunofluorescence microscopy after transduction of wild-type NPM-ALK or a kinase-negative mutant. Expression of wild-type NPM-ALK resulted in diffuse distribution of TRAF2 in the cytoplasm, whereas TRAF2 showed cytoplasmic and juxtamembrane aggregation in the presence of a kinase-negative NPM-ALK (Figure 2C). Taken together, these results support the hypothesis that NPM-ALK inhibits the CD30-NF-κB signaling pathway by blocking recruitment and aggregation of TRAF proteins.

To investigate whether the CD30-NF-κB signaling pathway is affected downstream of TRAF proteins in ALCL cells, we next examined NF-κB activation by overexpression of TRAF proteins. When Karpas 299 and SUDHL-1 ALCL cells were used, overexpression of TRAF proteins activated the NF-κB-driven promoter 3- to 5-fold, as was observed in other cell lines (Figure 2D). Furthermore, transduced TRAF2 protein showed cytoplasmic aggregation in these cells (Figure 2E). Thus, overexpression of TRAF proteins in ALCL cells can activate NF-κB by overcoming the inhibitory activity of NPM-ALK. These results indicate that the signal transduction pathway downstream of TRAF proteins is not affected in these ALCL cell lines.

Interaction of NPM-ALK with TRAF proteins

We next tested the possibility that NPM-ALK may inhibit recruitment and aggregation of TRAF proteins by binding to them. Coimmunoprecipitation studies of ALCL cell lines demonstrated association of endogenous NPM-ALK with TRAF2 (Figure 3A), as well as with TRAF5 (data not shown). We next tried to clarify the domain of NPM-ALK required for this association. Using expression vectors for NPM-ALK and a series of NPM-ALK mutants, including a kinase-negative mutant of NPM-ALK, association of these proteins with TRAF2 was examined. Anti-TRAF2 antibody coimmunoprecipitated both the wild-type and kinase-negative NPM-ALK. The ALK domain alone was also coimmunoprecipitated with TRAF2, whereas the NPM domain was not (Figure 3B). Thus, NPM-ALK appears to interact with TRAF2 at the ALK domain, irrespective of its kinase activity.

Coimmunoprecipitation studies using C-terminally deleted NPM-ALK mutants revealed that the ALK kinase domain is required for association with TRAF2 (Figure 3C). Thus, we next searched for the consensus TRAF binding motif (Ye et al., 1999) within the kinase domain of ALK and found a motif of SNQE (394–397 aa of NPM-ALK). Coimmunoprecipitation studies using a mutant of the TRAF binding domain demonstrated loss of association between TRAF2 and NPM-ALK (Figure 3D). Taken together, these results revealed that TRAF2 associates with NPM-ALK at the consensus binding motif located in the ALK kinase domain.

As shown above, NF-κB activation induced by CD30 overexpression was inhibited by coexpression of wild-type NPM-ALK but not by a kinase-negative mutant of NPM-ALK (KN-NPM-ALK) (Figure 2B). We next studied functional effects of the NPM or ALK domains on the NF-κB-driven promoter. Expression of the NPM domain alone did not show a significant suppression of NF-κB-driven promoter activities, nor did the ALK domain alone (Figure 3E). Similarly, expression of a TRAF binding-deficient mutant of NPM-ALK did not show interference with CD30-mediated activation of NF-κB (data not shown). These results indicate the interaction of ALK with TRAF proteins is not sufficient to inhibit CD30 signaling, but suggest that both the kinase activity and the chimeric structure of NPM-ALK are required for the inhibition.

ALK chimeras other than NPM-ALK cannot inhibit NF-κB activation

Variants of the ALK chimeric protein, TPM3-ALK and TFG-ALK, have been reported in a minority of ALCL (Hernandez et al., 1999; Lamant et al., 1999). The ALK of these chimeras is believed to be activated by oligomerization through the partner domains. To elucidate the functional roles of the NPM domain, we next studied the activities of these variant ALK chimeras on

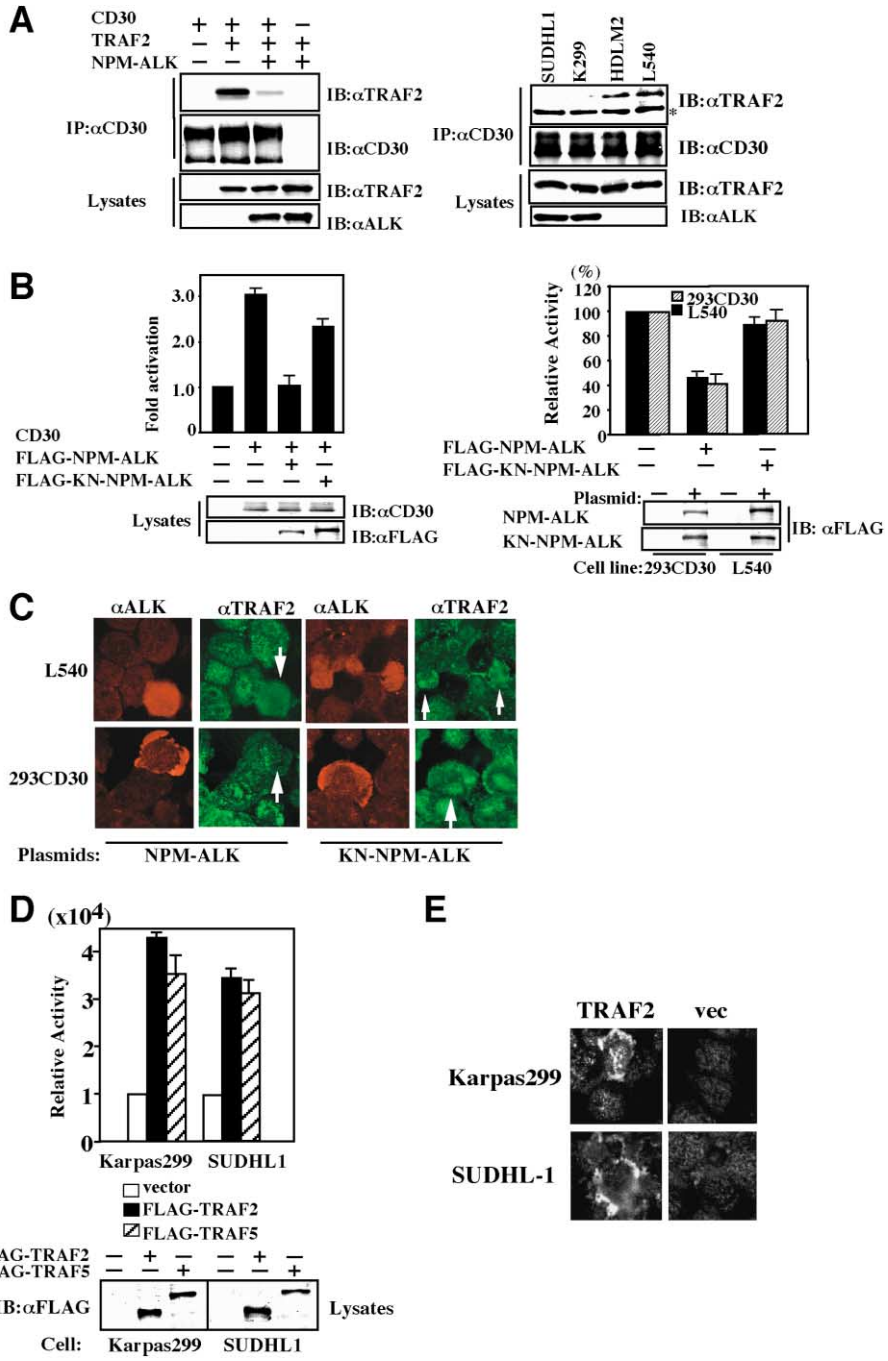

Figure 2. Inhibition of CD30 signaling by NPM-ALK

A: NPM-ALK inhibits interaction between CD30 and TRAF proteins. Upper panels, immunoprecipitates with anti-CD30 antibody were blotted by anti-TRAF2 or anti-CD30 antibodies; lower panels, whole cell lysates were blotted with antibodies against proteins indicated on the right. Left panel, coimmunoprecipitation analysis of transiently overexpressed CD30 and TRAF2 using HEK293T cells. Right panel, coimmunoprecipitation analysis of endogenous CD30 and TRAF2. IP, immunoprecipitation; IB, immunoblotting; lysates, whole cell lysates. The asterisk indicates the position of the Ig heavy chain.

B: Reporter gene analysis with $[kB]_6$-Luc plasmid. Left panels, transient transfection assays in HEK293 cells. KN-NPM-ALK, a kinase negative mutant of NPM-ALK. Left upper panel, results of triplicate experiments are shown with the mean and standard deviation. A representative result of three independent experiments is presented. Left lower panels, immunoblot analysis of whole cell lysates with anti-CD30 or anti-FLAG M2 antibody. IB, immunoblotting; lysates, whole cell lysates. Right panels, transient transfection assays using cell lines with ligand-independent signaling of CD30. Right upper panel, results of triplicate experiments are shown with the mean and standard deviation. A representative result of four independent experiments is presented. L540, an H-RS cell line of T cell origin; 293CD30, a stable transformant of HEK 293 cells highly expressing CD30. Right lower panels, immunoblot analysis of whole cell lysates with anti-FLAG M2 antibody. IB, immunoblotting; lysates, whole cell lysates.

C: Analysis by confocal microscopy of distribution of endogenous TRAF2. Antibodies used are indicated on the top. Expression plasmids transfected are shown below the panel. Arrows show the cells expressing the transduced proteins.

D: Reporter gene assays with $[kB]_6$-Luc plasmid using ALCL cell lines. Upper panel, results of triplicate dual luciferase assays are shown. A representative result of three independent experiments is presented. Transfected expression plasmids are indicated below the graph. Lower panel, immunoblot analysis of whole cell lysates with anti-FLAG M2. IB, immunoblotting; lysates, whole cell lysates.

E: Analysis by confocal microscopy of TRAF2 distribution in ALCL cell lines transfected with TRAF2 expression vector.

the CD30 signaling pathway. In transient reporter gene assays using an NF-κB-driven luciferase construct and HEK293 cells, these chimeric ALK proteins did not inhibit NF-κB activation by CD30 (Figure 4A). Instead, transduction of TPM3-ALK or TFG-ALK enhanced NF-κB-driven luciferase activities about 2- to 3-fold compared with that due to CD30 alone. Lack of inhibitory activities of these chimeric proteins on NF-κB activation was also observed when they were transduced into L540 cells (Figure 4B) or 293CD30 cells (data not shown). We next examined whether these chimeric proteins can induce changes in the intracellular distribution of TRAF proteins. To this end, we studied by confocal immunofluorescence microscopy L540 and 293CD30 cells after transduction of NPM-ALK or TPM3-ALK protein. Cytoplasmic aggregation of TRAF2 did not show any changes in the presence of TPM3-ALK protein, whereas it disappeared in cells transduced with NPM-ALK (Figure 4C). Thus, NPM-ALK appears to inhibit NF-κB activation by blocking the aggregation of TRAF proteins, and other ALK-chimeras cannot inhibit NF-κB because of the lack of inhibition of TRAF aggregation.

Identification of the wild-type NPM as the kinase substrate of NPM-ALK

To elucidate the mechanism for inhibition of TRAF aggregation and signaling pathway by NPM-ALK, we next studied the target

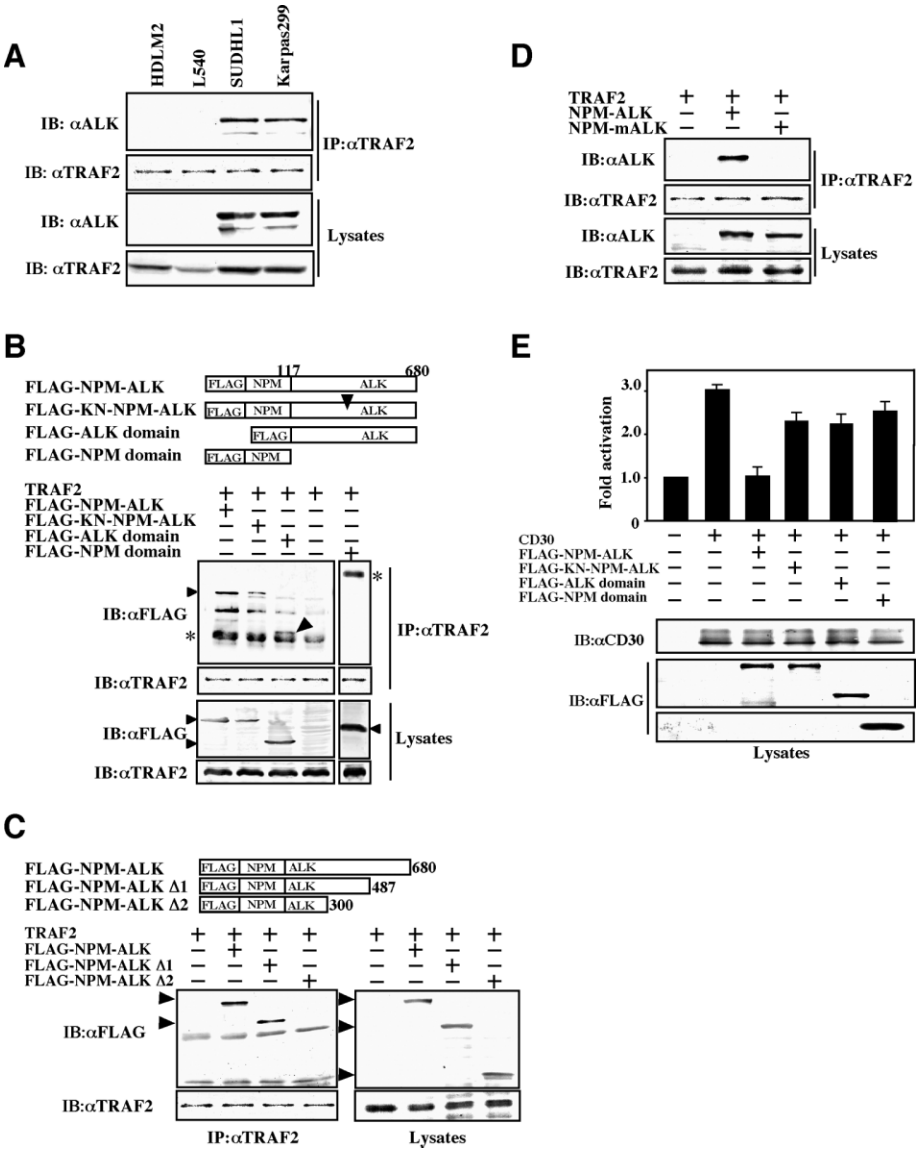

Figure 3. Association of NPM-ALK with TRAF2 and determination of TRAF2 binding region

A: Coimmunoprecipitation analysis of endogenous TRAF2 and NPM-ALK in ALCL cells. Upper two panels, immunoprecipitates of anti-TRAF2 antibody were blotted with anti-ALK or anti-TRAF2 antibody. Lower two panels, whole cell lysates were blotted by anti-ALK or anti-TRAF2 antibodies. IP, immunoprecipitation; IB, immunoblotting.

B and C: Analysis of the TRAF binding region in NPM-ALK. Results of coimmunoprecipitation studies of transiently overexpressed proteins in 293T cells are presented. Schematic figures of expression plasmids of FLAG-tagged proteins are presented above figures. Numbers indicate those of NPM-ALK amino acids. Arrowhead, kinase-negative mutation. **B:** Upper two panels, immunoprecipitates of anti-TRAF2 antibody were blotted with anti-FLAG M2 or anti-TRAF2 antibody. Lower two panels, whole cell lysates were blotted by M2 or anti-TRAF2 antibody. Right separated panels, NPM domain protein was separately analyzed with 10% SDS-PAGE. Arrowheads indicate positions of FLAG-tagged proteins. Asterisk shows the position of Ig heavy chain. IP, immunoprecipitation; IB, immunoblotting; lysates, whole cell lysates. **C:** Coimmunoprecipitation analysis with C-terminally deleted NPM-ALK proteins. Left lower panels, immunoprecipitates of anti-TRAF2 antibody were blotted by anti-FLAG M2 antibody (upper left panel) or anti-TRAF2 antibody (lower left panel). Right lower panels, expression of transduced proteins were confirmed by blotting of whole cell lysates with M2 antibody (upper right panel) or anti-TRAF2 antibody (lower right panel). Arrowheads indicate positions of FLAG-tagged proteins. IB, immunoblotting; lysates, whole cell lysates.

D: Coimmunoprecipitation analysis of a mutant NPM-ALK. NPM-mALK has a mutated TRAF binding consensus sequence in the kinase domain. Upper two panels, immunoprecipitates of anti-TRAF2 antibody were blotted by anti-ALK (top panel) or anti-TRAF2 antibody (second panel). Lower two panels, expression of transduced constructs was confirmed by blotting of whole cell lysates with anti-ALK (third panel) or anti TRAF2 antibody (bottom panel). IP, immunoprecipitation; IB, immunoblotting; lysates, whole cell lysates.

E: Reporter gene analysis with $[kB]_6$-Luc plasmid using HEK293 Jurkat cells. Top panel, results of dual luciferase assays of triplicate experiments are shown with the mean and standard deviation. A representative result of three independent experiments is presented. Lower three panels, immunoblot analysis of whole cell lysates with anti-CD30 (top of lower panels) or anti-FLAG M2 antibody (middle and bottom of lower panels). IB, immunoblotting; lysates, whole cell lysates.

of the NPM-ALK. Since we demonstrated interaction of TRAF2 with NPM-ALK (Figure 3), we first examined whether TRAF2 can be phosphorylated by NPM-ALK using in vitro kinase assays with anti-ALK antibody immunoprecipitates of HEK293T cells transfected by combinations of expression vectors for NPM-ALK, TRAF2, and wild-type NPM. Results did not show a band corresponding to phosphorylated TRAF2. Instead, a clear band of about 40 kDa was observed in the autoradiogram when wild-type NPM was cotransfected (Figure 5A, left panel), suggesting that wild-type NPM may be the kinase substrate of NPM-ALK. To test this possibility, we performed a kinase assay using a bacterially expressed recombinant NPM as substrate and an anti-ALK antibody immunoprecipitate of HEK293T cells transfected with an expression vector for NPM-ALK or a kinase-negative NPM-ALK. Results clearly showed that wild-type NPM can be phosphorylated by NPM-ALK (Figure 5A, right upper panel). Treatment of the gel with KOH did not abrogate phosphorylation (Figure 5A, right middle panel), excluding the possibility that the NPM may be phosphorylated by contaminated serine/threonine kinases.

Since the above results suggest that inhibition of TRAF2 association with CD30 may be mediated by functional modulation of the wild-type NPM by NPM-ALK, we next tested whether association between TRAF2 and CD30 can be inhibited by transduced wild-type NPM in the presence of NPM-ALK in a dose-dependent manner. Results of coimmunoprecipitation assays clearly demonstrated dose-dependent inhibition by transduced NPM, but no inhibition was observed by transduction of a

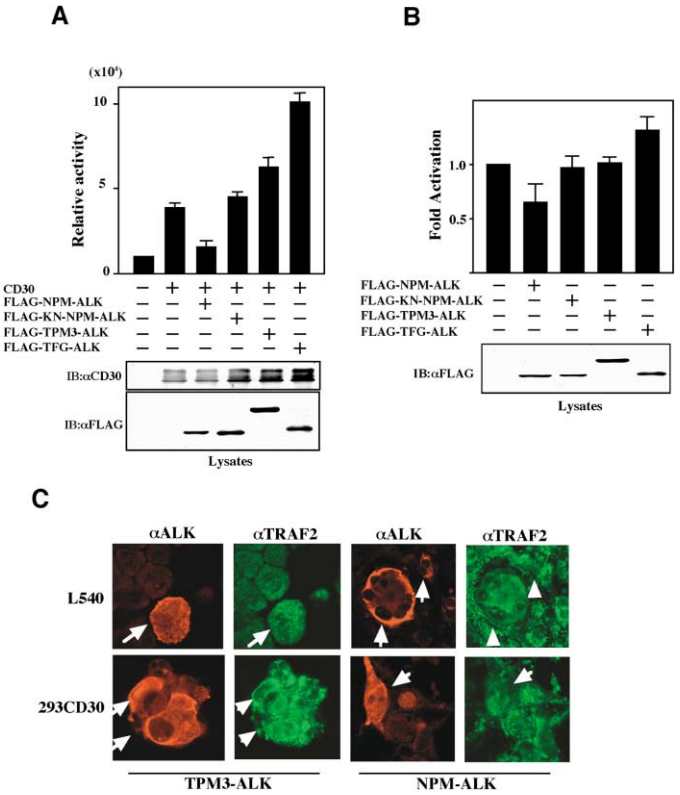

Figure 4. Absence of inhibitory activity of variants of ALK chimeras on CD30 signaling

A and B: Upper panels of **A** and **B**, reporter gene assays in HEK293 (**A**) and L540 (**B**) cell lines. Results of dual luciferase assays of triplicate experiments are shown with the mean and standard deviation. A representative result of three independent experiments is presented. Lower panels of **A** and **B**, immunoblot analysis of whole cell lysates. IB, immunoblotting; lysates, whole cell lysates.

C: Analysis by confocal microscopy of endogenous TRAF2 distribution. Antibodies used are indicated on the top. Expression plasmids transfected are shown below the panel. Arrows show the cells expressing the transduced proteins.

kinase-negative NPM-ALK (Figure 5B). Next, we examined whether the wild-type NPM is associated with NPM-ALK. Coimmunoprecipitation assays demonstrated strong association of NPM with NPM-ALK, whereas the kinase-negative NPM-ALK showed a decreased level of association (Figure 5C). Association between the endogenous wild-type NPM and NPM-ALK was also demonstrated in ALCL cell lines (Figure 5D), which excluded the possibility that the association was an artifact caused by overexpression of transduced proteins.

Phenotypic characteristics of stable transformants of H-RS cell lines expressing NPM-ALK

Since the above results suggested that expression of NPM-ALK may be critical to determine the phenotype of CD30-overexpressing cells, we next examined whether stable transduction of NPM-ALK in H-RS cell lines can induce phenotypic characteristics similar to ALCL cells in the transformants. Two H-RS cell lines of T lymphocyte origin, L540 and HDLM2, were stably transduced with NPM-ALK using a retrovirus vector. May-Gruenwald Giemsa staining of the transformants revealed marked changes in morphology compared with that of infected control cells. NPM-ALK transformants appeared larger in size and showed more abundant basophilic cytoplasm with numerous vacuoles, and prominent nucleoli. These characteristics are shared with ALCL cell lines, Karpas 299 and SUDHL-1 (Figure 6A). Next, we studied these NPM-ALK transformants for expression of ALCL marker proteins such as EMA and Clusterin (Jaffe, 2001). Laser confocal immunofluorescence microscopy clearly demonstrated induction or upregulation of EMA and Clusterin in these transformants (Figure 6B, upper panels). These marker proteins were shown to be expressed in a very similar pattern to that of ALCL cell lines (Figure 6B, lower panel). Immunoblot analysis of HDML2 transformants confirmed induction of EMA and Clusterin (Figure 6C). The same results were obtained when we characterized NPM-ALK transformants of a Mac1 cell line that is a cutaneous ALCL-derived T cell line with CD30 overexpression and without NPM-ALK (data not shown and Willers et al., 2003). The results suggest that phenotypic changes induced by NPM-ALK are not limited to HL-derived T cell lines, but are observed in other CD30 highly expressing T cell lines from ALCL without NPM-ALK.

Characteristics of signal transduction in NPM-ALK transformants

We next studied changes in the CD30 signal transduction in NPM-ALK transformants. As expected, the transformants showed loss of NF-κB binding activity in EMSA, whereas those infected with a vacant retrovirus vector clearly showed strong binding activity of NF-κB (Figure 7A, top panel). No significant differences in the levels of CD30 expression were observed among these cells (Figure 7A, bottom panel). Disappearance of TRAF protein aggregation in the cytoplasm was found in the NPM-ALK transformants, but not in control cells infected with a vacant retrovirus vector. Diffuse distribution of TRAF proteins in the cytoplasm of H-RS cell transformants was similar to that in ALCL cell lines (Figure 7B). NPM-ALK was expressed in the cytoplasm and nucleus in the transformants, as is observed in ALCL cell lines (Figure 7C). The results provide further support for the hypothesis that TRAF protein aggregation results from signaling of overexpressed CD30 as previously reported (Horie et al., 2002a). Next, we examined responses of the NPM-ALK transformants to CD30 stimulation by CD30 ligand (CD30L). They showed suppression of cell growth after CD30 ligand stimulation as is observed in Karpas 299 (Figure 7D). The results demonstrated that NPM-ALK inhibition of CD30-TRAF-NF-κB signaling pathway results in clear differences in biological effects of CD30 stimulation, and provide an explanation for the molecular basis of the previously described differences in responses to CD30L stimulation between HL and ALCL cell lines (Mir et al., 2000).

Discussion

In the present study, we demonstrate a unique function of the chimeric tyrosine kinase, NPM-ALK, that is expressed in most ALCL cases. NPM-ALK interferes with CD30 signaling through blocking recruitment and aggregation of TRAF proteins. NPM protein is shown to be phosphorylated by and form complexes with NPM-ALK. We further found that transduction of NPM-ALK into H-RS cell lines of T cell origin can bestow upon them ALCL-like morphology and induce expression of ALCL marker

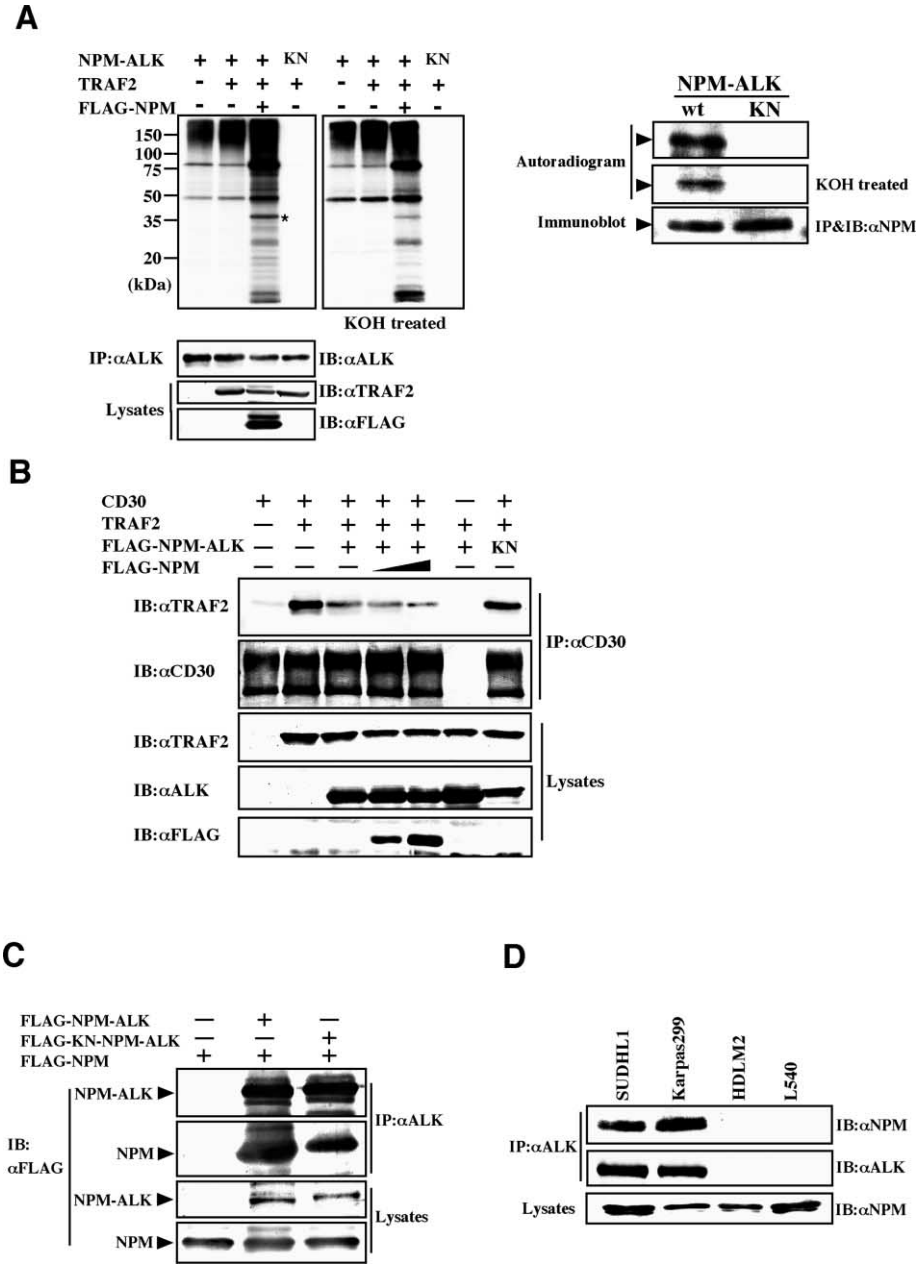

Figure 5. Phosphorylation of NPM by NPM-ALK and association of NPM with NPM-ALK

A: In vitro kinase assays. Left upper two panels, autoradiogram of kinase assays. Anti-ALK antibody immunoprecipitates of HEK293T cells transfected by expression plasmids indicated above the figure were used for a kinase reaction. Asterisk indicates the position of the wild-type NPM protein. Left lower three panels, immunoblot analysis of anti-ALK antibody immunoprecipitates (top panel) and whole cell lysates (middle and bottom panels) with antibodies indicated on the right. KN, a kinase negative NPM-ALK. Right panels, results of an in vitro kinase assay using a bacterially expressed His-tagged wild-type NPM as a substrate. The wild-type or kinase negative mutant NPM-ALK was expressed in HEK293T cells. Top and middle of right panels, autoradiograms of the kinase reaction mixture before (top panel) and after (middle panel) KOH treatment of the gel, respectively. Bottom of right panels, immunoblot analysis of the kinase reaction mixture. KN, a kinase negative NPM-ALK; IP/IB, immunoprecipitation and immunoblotting.

B: Wild-type NPM potentiates inhibitory activity of NPM-ALK against interaction between CD30 and TRAF2. Upper two panels, coimmunoprecipitation analysis. Anti-CD30 antibody immunoprecipitates of HEK293T cells transfected with plasmids indicated above were blotted with anti-TRAF2 (top panel) or anti-CD30 antibody (second panel). Lower three panels, immunoblots of whole cell lysates with antibodies indicated on the left. KN, expression plasmid for a kinase negative NPM-ALK. Closed triangle, 5 and 10 μg of FLAG-NPM. IP, immunoprecipitation; IB, immunoblotting.

C: Association of the wild-type NPM protein with NPM-ALK. HEK293T cells were transfected by plasmids indicated above the figure. Upper panels, immunoblot analysis anti-ALK antibody immunoprecipitates. Lower panels, immunoblot analysis of whole cell lysates with anti-FLAG antibody. IP, immunoprecipitation; IB, immunoblotting; lysates, whole cell lysates.

D: Coimmunoprecipitation of endogenous wild-type NPM protein with NPM-ALK. Upper panels, immunoblot analysis of anti-ALK antibody immunoprecipitates. Bottom panel, immunoblot analysis of whole cell lysates. IP, immunoprecipitation; IB, immunoblotting; lysates, whole cell lysates.

proteins. Thus, our results suggest that expression of NPM-ALK in neoplastic T cells expressing high levels of CD30 may be a determinant of their phenotype as ALCL, whereas, in the absence of NPM-ALK, they may exhibit a phenotype of H-RS cells of Hodgkin's lymphoma.

Interference of NF-κB signaling

High levels of CD30 expression on tumor cells characterize both Hodgkin's lymphoma and ALCL. We previously showed that this overexpression of CD30 is the basis for constitutive NF-κB activation in H-RS cells through ligand-independent signaling (Horie et al., 2002b). Thus, absence of NF-κB activation in NPM-ALK-positive ALCL cells prompted us to analyze the function of NPM-ALK in the signal transduction of CD30.

The results of the present study clearly demonstrate NPM-ALK-mediated inhibition of the CD30-TRAF signaling pathway that is dependent on ALK kinase activity. The results also reveal a possible mechanism of inhibition. Recruitment and aggregation of TRAF proteins appear to be inhibited by chaperone activity of NPM that is incorporated in the macromolecular complex formed by oligomerization of NPM-ALK and wild-type NPM (Hingorani et al., 2000); NPM is a chaperone protein that can dissociate aggregated proteins. Association of NPM with NPM-ALK is significantly facilitated by tyrosine phosphorylation of NPM by NPM-ALK, which may explain the kinase activity-dependent inhibition of TRAF signaling. In this context, lack of inhibition by TPM3-ALK and TFG-ALK can be explained by their inability to block aggregation of TRAF proteins, since partners of these ALK chimeras do not have chaperone activity. Regulation of NPM chaperone activity through tyrosine phosphoryla-

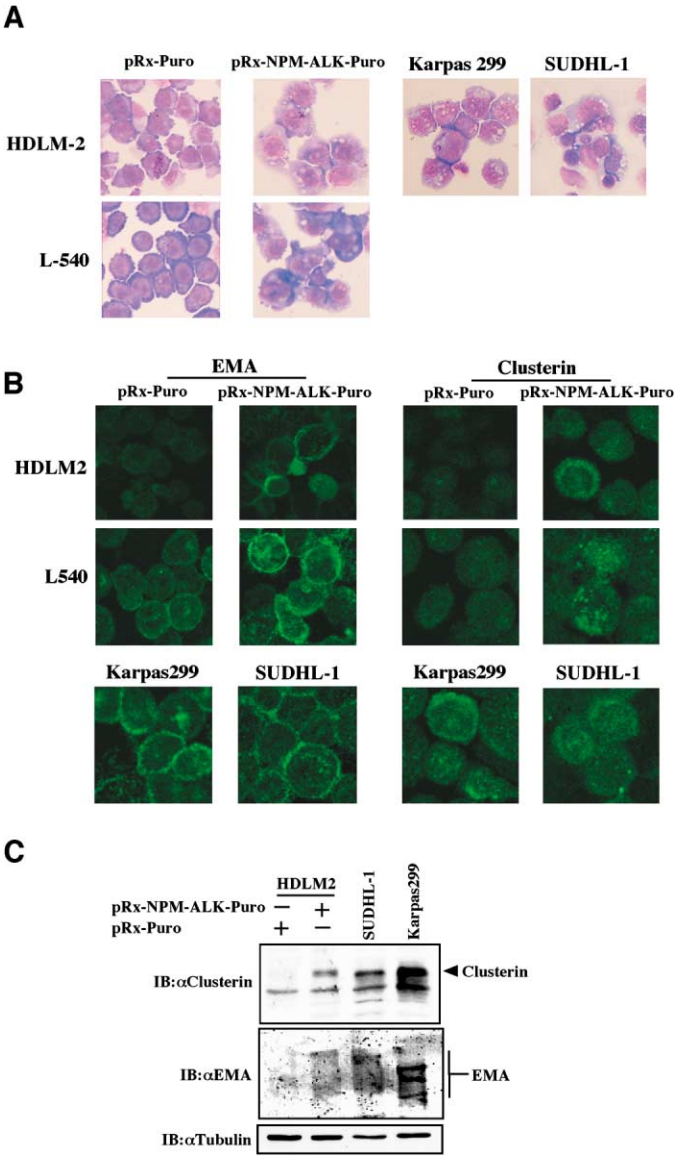

Figure 6. Phenotypic characteristics of stable transformants of H-RS cell lines expressing NPM-ALK

A: Photographs of cells stained with May-Gruenwald Giemsa. Magnification = 400×.

B and C: Induction of ALCL marker proteins in NPM-ALK-transduced transformants of H-RS cell lines. **B:** Upper panels, confocal immunofluorescence analysis of transformants. Parental cell lines are indicated on the left. Antibodies used are indicated above the photographs. Bottom panel, ALCL cell lines stained with antibodies indicated above. pRx-Puro, transformants infected with a vacant retrovirus vector; pRx-NPM-ALK-Puro, transformants infected with the retrovirus vector having NPM-ALK. **C:** Immunoblot analysis of marker proteins. HDLM2 cells infected with a vacant or NPM-ALK containing vector were studied for expression of marker proteins. Whole cell lysates were blotted with anti-clusterin (top panel), EMA (middle panel), and tubulin (bottom panel) antibodies.

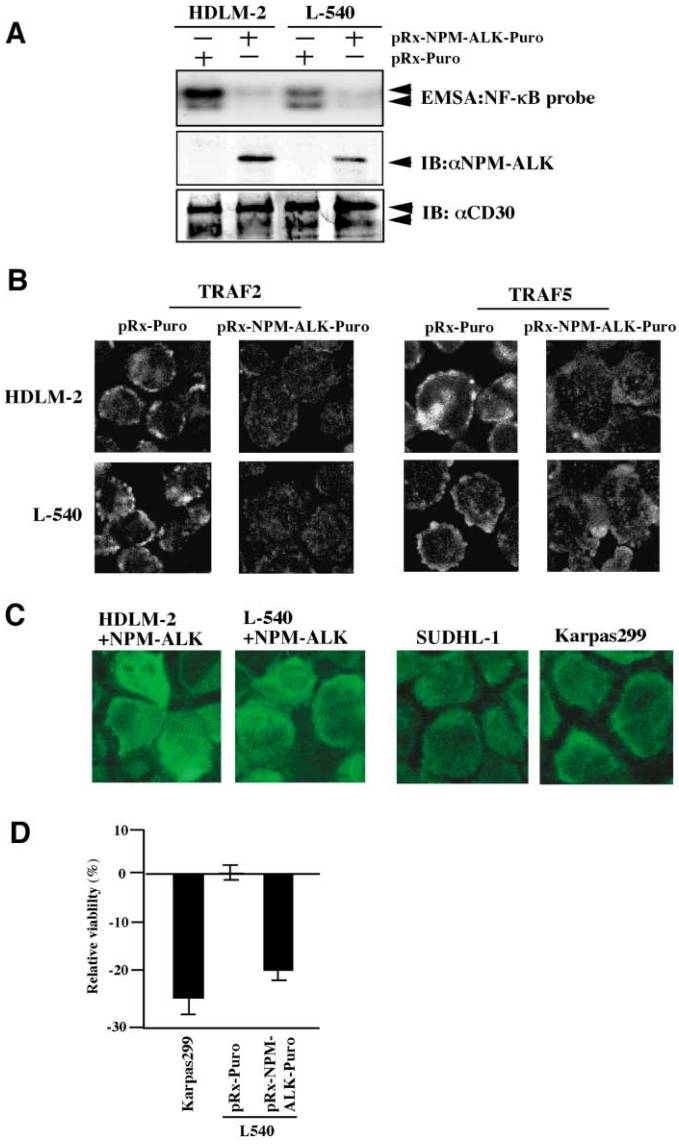

Figure 7. Alteration of CD30-TRAF signaling pathway in NPM-ALK transformants of H-RS cell lines

A: Absence of NF-κB activation in NPM-ALK expressing transformants. Top panel, EMSA analysis with NF-κB probe. Middle and bottom panels, detection of transduced NPM-ALK and CD30 proteins, respectively, by immunoblot analysis. IB, immunoblotting.

B: Confocal immunofluorescence analysis of TRAF protein distribution in transformants. SUDHL1 and Karpas 299 ALCL cell lines analyzed with anti-TRAF2 antibody; retrovirus vectors used are indicated above photographs; NPM-ALK, transformants infected with the retrovirus vector having NPM-ALK.

C: Confocal immunofluorescence analysis of NPM-ALK in transformants. SUDHL and Karpas 299 ALCL cell lines analyzed with anti-ALK antibody.

D: Cell kinetic analysis after CD30L stimulation. Relative levels of viable cell number examined by trypan blue dye exclusion test are presented. Bars indicate standard deviation (SD) of triplicate experiments.

tion by NPM-ALK is a novel finding, although it has previously been reported that functions of NPM can be modulated by various kinases such as CDK2/cyclin-E (Okuda et al., 2000) and CK2 (Szebeni et al., 2003).

We further demonstrated binding of TRAF2 with NPM-ALK through a consensus binding motif in the kinase domain of ALK (amino acids 394 to 397 of NPM-ALK). However, its functional significance is not entirely clear at present, since binding is not dependent on the kinase activity of ALK. Furthermore, TRAF2 does not appear to be the primary target of NPM-ALK, since we could not demonstrate increased levels of tyrosine phos-

phorylation of TRAF proteins by immunoblotting with an anti-phosphotyrosine antibody (data not shown) or an in vitro kinase assay using [γ-^{32}P] ATP (Figure 5A).

Hubinger et al. reported that NPM-ALK interacts with the cytoplasmic tail of CD30 through the ALK domain (Hubinger et al., 2001), suggesting a regulatory function of NPM-ALK on CD30 signaling. However, the association between NPM-ALK and CD30 does not appear to be involved in the inhibition of NF-κB, since it was dependent on the NPM domain (Figures 3E, 4A, and 4B).

Our study can help to explain the divergent effects of CD30 crosslinking previously reported in Hodgkin's lymphoma and ALCL (Hubinger et al., 2001; Levi et al., 2001; Schneider and Hubinger, 2002; Willers et al., 2003). In general, CD30 crosslinking caused increased proliferation of T cell type Hodgkin lymphoma cell lines but caused apoptosis or cell cycle arrest of NPM-ALK-positive ALCL cells. In contrast, CD30 crosslinking resulted in NF-κB activation and stimulation of NPM-ALK negative cutaneous ALCL.

Collectively, our results reveal a novel and unique function of the chimeric tyrosine kinase oncoprotein, NPM-ALK, although further studies are required for detailed characterization of the molecular mechanisms for regulation of the NPM chaperone activity and the functional significance of TRAF2 association with NPM-ALK.

Induction of phenotypic changes similar to ALCL cells

Our results show that transduction of NPM-ALK into H-RS cells of T-cell origin provides them with an ALCL-like morphology associated with induction of expression of ALCL marker proteins EMA/MUC1 and Clusterin/ApoJ. It has been established that expression of EMA/MUC1 and Clusterin/ApoJ characterizes ALK-positive ALCL (Jaffe, 2001). Detection of these two molecules on tumor cells is important for the pathologic diagnosis and classification of lymphomas. However, regulatory mechanisms for their gene expression in ALCL cells are largely unknown.

EMA/MUC1 is a highly glycosylated type I membrane glycoprotein that is abundantly expressed on the cell surface of many human adenocarcinomas like breast and ovarian cancers, and in B cell neoplasms such as multiple myeloma and B cell non-Hodgkin's lymphoma (Dyomin et al., 2000; Treon et al., 1999). Regulatory mechanisms of EMA/MUC1 expression have not been characterized, although involvement of tyrosine kinases such as c-src and ErbB2 has been suggested (Gonzalez-Guerrico et al., 2002; Scibetta et al., 2001). Clusterin/ApoJ was recently identified as a marker protein specifically expressed in systemic ALCL cells (Wellmann et al., 2000), although the specificity of its expression has been questioned by recent reports (Lae et al., 2002; Saffer et al., 2002). Again, there has been no clue to understand the mechanisms of Clusterin/ApoJ expression.

In the present study, we demonstrate that stable transduction of NPM-ALK into T cell-derived H-RS cell lines that were negative for these proteins induces expression of both genes, whereas induction was not observed in NPM-ALK transformants of L428, an H-RS cell line of B cell origin in which NF-κB activity was significantly suppressed (data not shown). The data suggest that the induction of EMA and Clusterin/ApoJ results from inhibition of TRAF signaling and introduction of the constitutively active tyrosine kinase, ALK. Abrogation of TRAF signaling alone does not appear to be sufficient, since TRAF2 overexpression in the NPM-ALK transformants did not show phenotypic reversion (data not shown). Since no detailed characterization of promoter activities of these genes is available at present, precise mechanisms of induction remain to be fully elucidated.

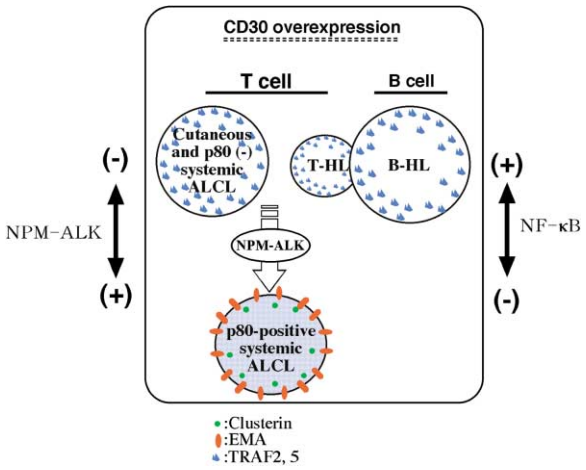

Figure 8. Schematic view of NPM-ALK function in ALCL cells

Presence of p80 NPM-ALK in CD30 overexpressing neoplastic T cells can determine their phenotype through interference with the CD30-TRAF-NF-κB signaling pathway.

Our results indicate that the presence or absence of NPM-ALK chimeric protein in CD30-overexpressing neoplastic T cells has a determining effect on their phenotype. These results provide a novel insight into understanding the molecular basis of pathophysiology of these lymphomas and may have an impact on the classification of CD30-positive lymphomas (Figure 8). There are three categories of lymphoma with closely related phenotype: Hodgkin's lymphoma, ALK-positive and ALK-negative systemic (or nodal) ALCL, and ALK-negative cutaneous T cell lymphoma. Tumor cells of the latter two categories are of T cell origin (Stein et al., 2000). All these lymphomas are characterized by high levels of CD30 expression. A great majority of H-RS cells originate from germinal center B cells, with a minority of T cell origin H-RS cells (Kadin et al., 2001; Muschen et al., 2000; Seitz et al., 2000). Thus, our results suggest the possibility that NPM-ALK-positive ALCL represents a unique entity, whereas there might be no clear distinction between HL of T cell origin, ALK-negative systemic ALCL, and ALK-negative cutaneous T cell ALCL.

Experimental procedures

Cell cultures

Jurkat, HEK293, and HEK293T cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and Fujisaki Cell Biology Center (Okayama, Japan). H-RS cell lines, HDLM-2 and L-540, and ALCL cell lines, SUDHL-1, Karpas 299, and SR786 were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cutaneous ALCL cell line Mac1 was described previously (Willers et al., 2003). Nonadherent cell lines were cultured in RPMI 1640 and adherent cells in DMEM with supplementation of recommended concentrations of fetal calf serum (FCS) and antibiotics. An HEK293 transformant (293CD30) was described previously (Horie et al., 2002a, 2002b).

Plasmids and cDNA

Expression vectors for human CD30 and FLAG-tagged TRAF2 and TRAF5 were described elsewhere (Aizawa et al., 1997; Horie et al., 1996, 1998). A cDNA clone for NPM-ALK was obtained by screening of a cDNA library prepared from Karpas 299 using a probe of ALK cDNA fragment amplified by reverse transcription PCR (RT-PCR). Expression vectors for the wild-type and N-terminally FLAG-tagged NPM-ALK were prepared using pME18S (a gift from Dr. Maruyama at Tokyo Medical and Dental University) and FLAG-tagged expression vector (pME-FLAG), respectively. Those for the NPM or ALK domains of NPM-ALK, C-terminally deleted NPM-ALK mutants, and the FLAG-tagged wild-type NPM were prepared using PCR-amplified fragments. Expression vectors for the TPM3-ALK or TFG-ALK fusion proteins were prepared by introducing PCR-amplified TPM3 or TFG fragments just upstream of ALK domain of pFLAG-ALK. The resultant plasmids were named pFLAG-TPM3-ALK and pFLAG-TFG-ALK. An expression vector for a kinase negative mutant of NPM-ALK (Y156/567F), pME-FLAG-KN-NPM-ALK, was described previously (Fujimoto et al., 1996) and is a gift from Dr. Yamamoto, The Institute of Medical Science, The University of Tokyo. A FLAG-tagged expression vector for a mutant NPM-ALK having point mutations in the putative TRAF binding motif, S394A/Q396A/E397A, was prepared by the method of Kunkel (1985). Primer information is shown in the Supplemental Data at http://www.cancercell.org/cgi/content/full/5/4/353/DC1.

Immunohistochemistry

Immunohistochemical and immunofluorescence analyses of cultured cells and biopsied lymph nodes were done as described (Horie et al., 2002b), and visualized with a confocal microscope (Radiance 2000, BioRad). Antibodies used are listed in Table S2 of the Supplemental Data. To detect TRAF and CD30 proteins in lymph node samples, a modification of the tyramide signal amplification (TSA) system (NEN Life Science) was used in order to facilitate use of streptavidine-FITC or streptavidine-Texas Red instead of peroxidase-conjugated streptavidine. Antibody information is shown in the Supplemental Data.

Immunoblotting

Immunoblotting experiments and coimmunoprecipitation were done as described (Horie et al., 1996). Transfection was done by Lipofectin reagent (Invitrogen) using 2×10^6 HEK293T cells, and total of 2 μg of expression vectors. A vacant expression vector pME18S was used in control transfections or to make the total amount of transfected plasmid 2 μg. Antibody binding was detected using ECL chemiluminescence kits (Amersham) or Western blue stabilized substrate for alkaline phosphatase (Promega). Antibody information is shown in the Supplemental Data.

In vitro kinase assay of NPM-ALK

Wild-type or the kinase-negative NPM-ALK was expressed in HEK293T cells and immunoprecipitated using anti-ALK antibody (MBL, Nagoya, Japan). Bacterially expressed His-tagged wild-type NPM protein was prepared using pBAD Directional TOPO Expression system (Invitrogen, Carlsbad, CA) and used as a substrate for the in vitro kinase assay. Kinase reaction was done as described (Fujimoto et al., 1996). The NPM protein in the reaction mixture was immunoprecipitated with anti-NPM antibody (Santa Cruz Biotech, CA). Tyrosine phosphorylation was confirmed by autoradiography after treatment in 1N KOH at 60°C for 90 min.

Reporter gene assays

Activation of NF-κB was tested by reporter gene assays, using a κB-site dependent luciferase vector, $[kB]_6$TK-Luc, as described elsewhere (Horie et al., 1998). Renilla luciferase expression vector, pRL-TK (Promega, Madison, WI), was cotransfected to standardize each experiment. Luciferase activity was measured by Dual Luciferase assay kit (Promega).

Electrophoretic mobility shift analysis (EMSA)

Detection of NF-κB by EMSA was done as described (Horie et al., 1998). Nuclear extracts were prepared according to the method of Andrews and Faller (1991).

Northern blot

Northern blot analysis was carried out essentially as described (Horie et al., 1996; Watanabe et al., 2003), using 2 μg/lane of poly (A)-selected RNA per lane. Probes used were as follows: a 980 bp of CD30 and 838 bp of GAPDH cDNA fragments amplified by RT-PCR and cloned in pGEM-T vector (Promega).

Retrovirus-mediated transduction of NPM-ALK to H-RS cell lines

The NPM-ALK cDNA containing the entire coding region was cloned into retrovirus vector pRx-puro (Wakimoto et al., 1997) and the resultant plasmid was named pRx-NPM-ALK-puro. Virus-containing supernatants were prepared using the Phenix-Ampho packaging cell line. Control experiments using pRx-GFP-Puro vector showed transduction efficiency of more than 20% for both cell types (data not shown). Transfectants were selected using 2 μg/ml puromycin (SIGMA). In most transduction experiments, about one-third of cells appeared to survive puromycin selection. NPM-ALK expression was examined by immunoblotting out as described previously.

Cell kinetic analysis

Two 96-well flat-bottomed microtiter plates (Coaster 3361 high binding) were coated with 50 ng of a human recombinant CD30L (ALEXIS Biochemicals San Diego, CA). After overnight incubation of the plates at 4°C, the coating solution was removed, and 100 μl culture medium containing 20,000 cells in logarithmic growth conditions was added to each well. To prevent further binding between soluble CD30 and CD30L, the cells were washed and fresh culture medium added at the time of transfer. The plates were incubated at 37°C in a 5% CO_2 atmosphere. After 48 hr, cells were stained by trypan blue solution (Biochrom KG, Berlin, Germany) and viable cells were counted.

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