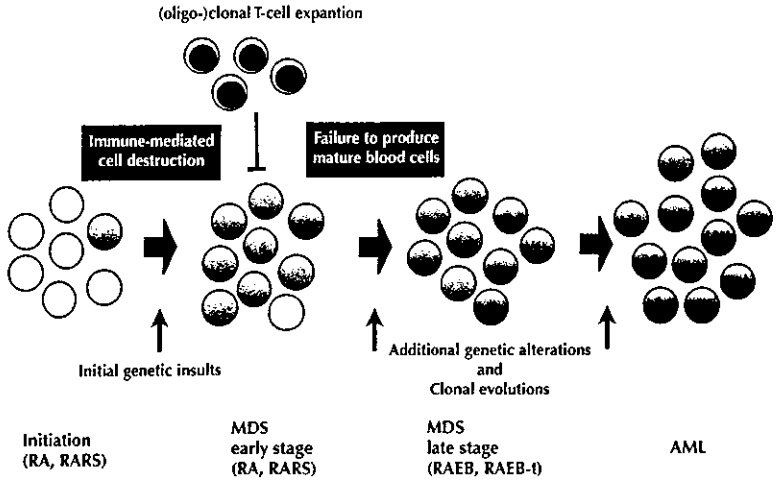

Figure 1. Multistep model of MDS pathogenesis

and pathophysiologic implications, that significant residual normal hematopoiesis could be still detected in many MDS bone marrows[12].

Multistep model of development and progression of MDS

A multistep model of genetic alterations has been frequently employed to explain the pathogenesis and development of many neoplastic disorders, including MDS. It is mainly based on temporal profiles of cytogenetic as well as other genetic abnormalities during courses of MDS, which often show emergence of new subclones having additional chromosomal abnormalities and later expansion of these subclones (Figure 1). However, it is not clear how many and what kind of genetic changes are required for development or transformation of MDS. There is no definitive evidence that multiple genetic insults are really required for development of MDS. In addition, although a number of genetic alterations have been reported in MDS patients as described below, the majority of the currently identified genetic abnormalities are found both in MDS and AML or preferentially observed in advanced or transformed cases of MDS. Thus it should be stressed that the early genetic alterations in MDS are mostly unknown.

Genetic abnormalities in MDS

A wide variety of genetic alterations have been described in MDS, including point mutations and generation of aberrant fusion genes associated with recurrent balanced translocations. A list of these abnormalities, not complete through is given in Table 1.

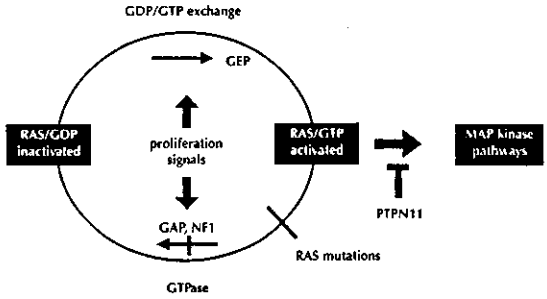

Figure 2. Abnormalities in the *Ras* pathways in *MDS*

Mutations of *RAS*, *p53*, and other genes in MDS

Among these genetic alterations, the first identified are mutations of the *N-RAS* proto-oncogene[13]. RAS is a key molecule for the MAP kinase cascade to transduce proliferation signals to nucleus. Mutations exclusively occur at codon 12, 13 or 61, which converts RAS to a constitutive active molecule (Figure 2). In contrast to early reports showing mutation rates of 30 to 40%, more recent studies reported much lower frequencies of ~10% on average[14]. *p53* is another target for mutations in MDS[15]. *p53* is the most frequently inactivated tumor suppressor gene (TSG) in human cancers and its diverse functions have been extensively studied, although the frequencies of its mutations are generally lower in hematopoietic tumors. Referring to the TP53 database, 82 of 646 (~12%) MDS cases are reported to have mutated *p53* genes (http://www.iarc.fr/p53/), but this figure may provide too high an estimation, probably suffering from a publication bias. Both *RAS* and *p53* mutations are rare in early stages

Table 1. Genetic abnormalities in MDS

Mutation, Deletion, Duplication		
N-RAS	~10%	mutation
NF1	childhood MDS, JMML	mutation
PTPN11	childhood MDS, JMML	mutation
p53	<10%	mutation, deletion
FLT3	~5%	tandem duplication
FMS	rare	mutation
KIT	rare	mutation
AML1	~10%	mutation
ATRX	rare AT-MDS	mutation
TERC	rare	mutation
Cytochrome c	rare	mutation
WT1	rare	mutation
Gene Rearrangements		
EVI1(3q26) and MEL1(1p36)		
t(3;3)(q21;q26)	*EVI1 overexpression*	
inv(3)(q21q26)	*EVI1 overexpression*	
t(3;21)(q26;q22)	*AML1/EVI1*	
t(3;12)(q26;p13),	*TEL/EVI1*	
t(1;3)(p36;q21)	*MEL1 overexpression*	
TEL/ETV6(12p13)		
t(5;12)(q33;p13)	*TEL/PDGFR-b*	
t(9;12)(q22;p12)	*TEL/SYK*	
t(1;12)(36.1;p13)	*TEL/MDS2*	
t(5;12)(q31;p13)	*ASC2/TEL*	
t(12;22)(p13;q11)	*MN1/TEL*	
MLL(11q23)		
t(11;16)(q23;p13)	*MLL/CBP*	
t(11;19)(q23;p13.1)	*MLL/MEN*	
t(5;11)(q31;q23)	*MLL/GRAF*	
NUP98 and CAN		
t(7;11)(p15;p15)	*NUP98/HOXA9*	
inv(11)(p15q22)	*NUP98/DDX10*	
t(2;11)(q31;p15)	*NUP98/HOXD13*	
t(11;17)(p15;q21)	*NUP98/HOXB*	
t(11;12)(p15;q13)	*NUP98/HOXC13*	
t(11;20)(p15;q11)	*NUP98/TOP1*	
t(6;9)(p23;q34)	*DEK/CAN*	
Other		
t(3;5)(q25;q34)	*NPM/MLF-1*	

of MDS (RA/RARS) and typically found in more advanced stages (RAEB/RAEBt) or during transformation to AML[16].

Activated mutations of receptor tyrosine kinases have been also reported in MDS, including mutations of the *c-FMS* gene encoding M-CSF receptor and of the *FLT3* gene[17-19]. Mutations of the *c-FMS* gene were reported to be more common in CMMoL but have not repeatedly confirmed, while the *FLT3* mutations are considered more important for the pathogenesis of AML or progression form MDS to AML rather than development of MDS itself. Mutations of the *Chk2* gene, and human telomerase RNA gene (*TERC*)[20] and deletion of α-*globin* gene clusters[21] are also found in isolated reports. Inactivation mutations of the *ATRX* gene are found in rare cases of AT-MDS characterized by myelodysplasia with severe microcytic anemia due to α-thalathmia[22]. ATRX is a SWI/SNF like protein and involved in transcriptional regulation of genes including the α-globing gene. Constitutional inactivation of the *ATRX* gene causes ATRX syndrome, a rare X-linked disorder showing a-thalathmia, mental retardation, facial dysmorphism, and urogenital abnormalities[23,24]. Since patients with ATRX syndrome do not develop MDS, acquired *ATRX* mutations in AT-MDS cases are not considered to play a role in initiation of MDS, but to modify its phenotype. Mutations in mitochondrial respiratory genes such as the *cytochrome c oxidase (CXO)* gene have been also reported in MDS patients in high frequencies[25]. Since defects in CXO will compromise sufficient oxidative energy production in mitochondria required for the driving force of mitotic spindles, it raises an attractive hypothesis that inactivation of the *CXO* gene will lead to genetic instability due to mitotic dysfunction[25], but the observation has not been firmly confirmed[26].

AML1/Runx1 gene

AML1 or *Runx1* is a well-known target of t(8;21)(q22;q22) translocation found in AML, in which *AML1* is rearranged with *ETO* to generate the *AML1/ETO* fusion gene[27]. *AML1* is also involved in t(12;21)(p13;q22) and t(3;21)(q26,q22) to form TEL/AML1 and AML1/Evi1 fusion protein[28,29], respectively. It encodes a transcription factor that regulates transcription of a wide variety of genes expressed in hematopoietic compartments and is indispensable for establishment of definitive hematopoiesis[30-32]. Importantly, a germ line mutation of *AML1* causes a rare hereditary disorder known as the familial platelet disorder with a predisposition to AML (FPD/AML)[33]. It typically has several years of a preleukemic period showing dysmegakaryopoiesis with reduced platelet counts and may be considered as a kind of congenital form of MDS, in which an *AML1* mutation is clearly the first and possibly sufficient genetic hit that contributes to its MDS-like phenotype. Of particular note is that acquired mutations of *AML1* are commonly found in sporadic cases of AML (53/619; 8.6%) especially of M0 phenotype (39/185; 21%), and also in MDS cases

(33/362; 9.1%) (Figure 3)[34-38]. An excellent review is available covering both sporadic and familial mutations of *AML1*[39]. *AML1* mutations may be hemi- or biallelic with a substantially higher biallelic mutation rate in the AML M0 subtype[34,38,40]. In MDS *AML1* mutations seems to be detected in more advanced stages (RAEB/RAEBt and MDS derived from AML) and in therapy related MDS (tMDS)[41]. Recently a strong association of *AML1* mutations with monosomy 7 has been reported in advanced stage MDS, suggesting cooperative roles between both abnormalities[37]. *AML1* mutations can be rarely detected in apparently healthy persons and frequently found in atomic bomb-related MDS cases. Thus *AML1* locus may be more prone to mutations[40,42,39]. Some mutations lead to simple loss-of-function proteins, while others clearly generate mutants having dominant-negative activities against the normal AML1 protein. Propensity to AML also differs among FPD/AML pedigrees and possibly among sporadic cases with different *AML1* mutations, depending on types of the mutations[33,43].

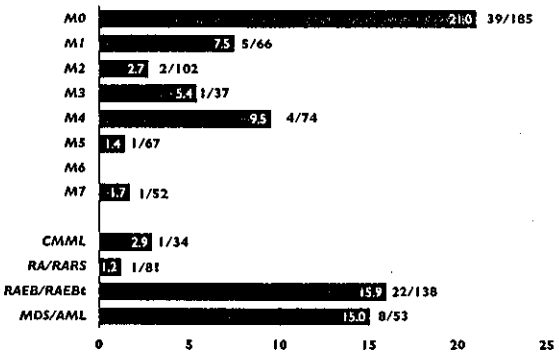

Figure 3. Frequencies of *AML1* mutations

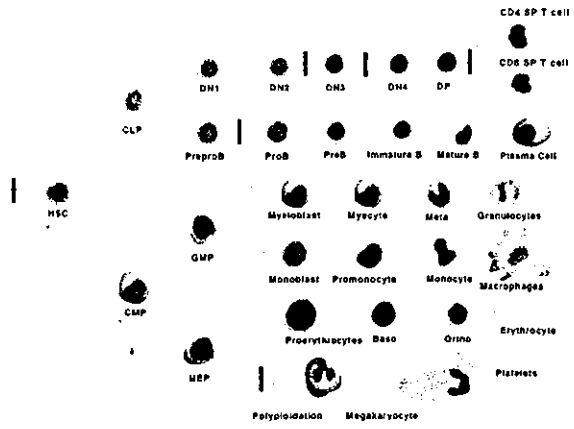

Figure 4. Loss of *AML1* blocks normal hematopoiesis at multiple stages

Several mouse models carrying abnormal *AML1* genes have been generated. *AML1*-null mice are embryonic lethal on E12.5 and *AML1/ETO*-knockin mice also show a similar phenotype, indicating that AML1-fusion protein act in a dominant negative manner[44,45,46]. Unexpectedly, however, when *AML1* is disrupted in adult bone marrow, hematopoietic stem cells are still maintained or rather increased in number, while severe dysmegakaryopoiesis prevails showing defects in polyploidation and reduced platelet counts. Intriguingly, production of both mature T cells and B cells is also severely impaired in the *AML1-null* mice (Figure 4)[47], which may give rise to a plausible explanation for frequent sparing of lymphoid lineages in MDS.

NF1 gene

The *NF1* gene was originally identified as the causative gene for neurofibromatosis type I (NF1) and encode neurofibromin, a GTPase-activation protein (GAP) for p21RAS[48,49]. *NF1* is one of the prototypes of a tumor suppressor gene, inactivation of which leads to constitutive activation of p21RAS (Figure 2)[50]. NF1 patients has a strong predisposition to developing Juvenile Myelomonocytic Leukemia (JMML) (~200 to 500 fold increase in relative risk to the normal population), an aggressive form of childhood MDS characterized by monocytosis, thrombocytopenia, splenomegaly, and malignant infiltration of the skin, lymph nodes, lungs, liver, and other organs. In a large series of JMML, 14% were found to have NF1[51]. In mouse models somatic inactivation of *NF1* in hematopoietic cells results in a progressive myeloproliferative disorder resembling JMML[52]. It should be noted that *NF1* is mutated and inactivated in sporadic cases of childhood MDS or JMML, especially in combination with monosomy 7[53], although *NF1* mutations is rare in adult cases. Oncogenic *RAS* mutations were also found in JMML in high frequency and restricted to cases without NF1, underscoring pathogenic importance of the RAS-activating pathway for the pathogenesis of JMML[54].

PTPN11 gene

PTPN11 encodes SHP-2 protein tyrosine phospatase and is congenitally mutated in Noonan syndrome[55], a developmental disorder with short stature, facial dysmorphia,

skeletal anomalies, and occasional development of JMML. Intriguingly, somatic mutations of the *PTPN11* gene were found in 10 of 62(16%) sporadic cases of JMML, 5 of 50 (10%) other childhood MDS cases, and 1 of 26 (3%) AML[56,57]. SHP-2 seems to act in the RAS/MAPK cascade, because mutant SHP-2-introduced cells showed sustained activation of ERK2 in response to EGF stimulation (Figure 2)[58]. Also supporting this is that mutations were not detected in JMML associated with NF1 cases and in those who had *RAS* mutations. Mutations occurred exclusively in childhood MDS with advanced diseases as well as JMML[57]. No *PTPN11* mutations have been reported in adult MDS[59].

Cytogenetic abnormalities in MDS

A huge number of cytogenetic abnormalities have been described in MDS and provide important clues to delineate molecular bases for MDS pathogenesis[60]. Frequent cytogenetic abnormalities found in MDS are listed in Table 2. Although many of these abnormalities are common to both MDS and AML, rarity of disease-specific balanced translocations and high frequencies of unbalanced abnormalities compared to *de novo* AML, are among prominent cytogenetic features of MDS. tMDS tends to have more complex chromosomal anomalies, higher rates of hypodiploid, 5q-/-5, and 7q-/-7, and lower frequencies of trisomy 8 and hyperdiploid than *de novo* MDS[61].

Among recurrent unbalanced abnormalities in MDS are trisomy 8, 7q-/-7, 5q-/-5, 20q-, 13q-, 12p-, and 17p-[62]. A predominance of loss of, rather than gain of genetic materials may indicate importance of inactivation of TSGs for the pathogenesis of MDS[61]. However, in spite of a great deal of effort in the field of molecular genetics, no relevant TSGs has been successfully identified within the recurrently deleted chromosomal segments, while a number of target genes were identified from the breakpoints analysis of recurrent reciprocal translocations found in MDS/AML. In addition to technical difficulties arising from a large size of involved chromosomal segments to be analyzed, possibilities of haploinsufficiency and multiple target genes with regard to MDS pathogenesis may complicate molecular analysis of putative TSGs from chromosomal deletions.

Table 2. Common cytogenetic abnormalities in MDS/tMDS

30-50% of primary and ~80% of secondary MDS have chromosomal abnormalities.

Numerical	Translocations	Deletions
+8 (~20%)	inv3 & t(3;3) (4%)	del 5q (~25%)
-7 (~15%)	t(1;7) (2%)	del 11q (~3%)
-5 (~7%)	t(1;3) (1%)	del 12q (~5%)
-Y (~8%)	t(6;9) (<1%)	del 20q (~6%)
-17 (~4%)	t(5;12) (<1%)	del 7q (~5%)
		del 17p (~4%)
		del 13q (2%)

Translocations

As mentioned above, balanced translocations are more characteristic features of *de novo* AML than of primary MDS. However, they clearly play important roles in progression of MDS into AML. *Evi1* and *MEL1*, *MLL*, and *TEL/ETV6* are among genes most frequently involved in these translocations.

3q21q26 syndrome and t(1;3)(p36.1;q21)

t(3;3)(q21;q26) and inv(3)(q21q26) are observed in ~2% and ~4% of MDS, respectively, and also found in a similar proportion of AML cases. Many of these cases have common features of multilineage dysplasia, a normal to elevated platelet count with increased dysplastic megakaryocytes, minimal or no response to chemotherapy, and poor prognosis[63]. The former two abnormalities in AML are known as 3q21q26 syndrome, in which overexpression of *Evi1* seems to be a common finding although there is a conflicting report[64]. In 3q21q26 syndrome, it is postulated that juxtaposition of the *Evi1* gene to the *ribophorin I (RPN1)* locus on 3q21 leads to aberrant *Evi1* expression (Figure 5)[65]. *Evi-1* was originally identified at the common retrovirus integration site in myeloid leukemia from AKXD inbred mice, and encodes a transcription factor having two zinc finger motifs[66]. Later an alternative splicing form of Evi1 with an additional N-terminal sequence, referred to as *MDS1/Evi1*, was revealed to have a PR domain similar to RIZ and RPDM1 proteins, which also have an alternative isoform that lacks a PR domain (Figure 5)[67]. In 3q21q26 syndrome, a shorter Evi1 isoform is exclusively expressed from the rearranged allele, in which the *MDS1* promoter is lost or located too distant (~500kb) from the putative enhancer element of *RPN1* by gene rearrangements (Figure 5)[68].

Evi1 is also involved in other translocations and transcriptionally activated as found in t(2;3)(p15;q26), t(3;7)(q26;q22), and (3;13)((q26;q13-14)[69-71]. In addition, aberrant fusion genes involving *Evi1* have been reported

in other myeloid neoplasms especially tMDS, tAML, and myeloid crisis of CML carrying t(3;21)(q26;q22) and t(3;12)(q26;p13), in which *AML1/Evi1* and *TEL(ETV6)/Evi1* are generated, respectively[29,72]. Moreover, increased *Evi1* expression is also observed in ~9% of other AML and MDS cases as well as CML BC cases without 3q26-involving translocations or inversions and related to poor prognosis, suggesting critical roles of *Evi1* in human myeloid leukemogenesis[73-76].

Evi1 is presumed to bind specific DNA sequences and act as a strong repressor, for example, of GATA-1. In other contexts, it enhances AP1 activity[77] and can transform Rat-1 fibroblast *in vitro*. It also binds to Smad3 and inhibit TGF β signaling[78], which may be mediated by a transcriptional corepressor, CtBP[79]. Evi1 was also shown to interact with HDAC-1[80], which could mediate the repressor function of Evi1. Differential functions of MDS1/Evi1 and Evi1 isoforms are implicated in leukemogenesis[81], but their leukemogenic roles seem to be still controversial.

t(1;3)(p36;q21) is another translocations found in rare (~1%) cases of MDS and AML with similar clinicopathologic features to 3q21q26 syndrome. Interestingly, *MEL-1* on 1p36 that is highly homologous to *Evi1* is translocated to the *RPN1* locus on 3q21 and transcriptinally activated[82]. MEL1 also has two alternative splicing forms that are closely related to Evi1 and MDS1/Evi1, and a smaller *MEL1* product lacking a PR domain (MEL1S) is preferentially expressed in t(1;3)(p36;q21) (Figure 5)[83]. Similarity in disease phenotypes and involved genes seems to strongly support the idea that these '*Evi1* family genes' are the *bona fide* targets of 3q21q26 syndrome and t(1;3)(p36;q21).

***TEL/ETV6* translocations**

TEL or ETV6 is an ETS-like transcription factor first identified at the 12p13 breakpoint of t(5;12)(q33;p13), and reported to be essential for development and maintenance of hematopoiesis as well as for megakaryopoiesis (Blood 102:131a, 2003). In this translocation, TEL is fused to PDGFR-β (platelet-derived growth factor β), resulting in TEL/PDGFR- β fusion protein. t(5;12)(q33;p13) is associated with a rare form of CMMoL showing myelomonocytic proliferations with frequent eosinophilia[84], and may be more properly grouped together with t(1;4)(q44;q12) in hypereosinophilic syndrome, and t(4;22)(q22;q11) and t(9;12)(q34;p13) in *Ph1* negative CML variants, in which FIP1L1/ PDGFR- α , and BCR/ PDGFR- α and TEL/ABL are generated, respectively[85-87]. *TEL* assumes a promiscuous feature to generate various fusion genes with different partner genes in a wide variety of hematopoietic neoplasms including MDS and AML derived from MDS[88]. *Syk*, *MDS2*, *Evi1*, *ASC2*, and *MN1* are among fusion partners of *TEL* gene in t(9;12)(q22;p12), t(1;12)(36.1;p13), t(3;12)(q26;p13), t(5;12)(q31;p13), and t(12;22)(p13;q11), respectively[72,89-92].

TEL seems to provide an interface for dimerization via its HLH domain and to activate fused kinases in some translocations, while in others, apparently functionless,

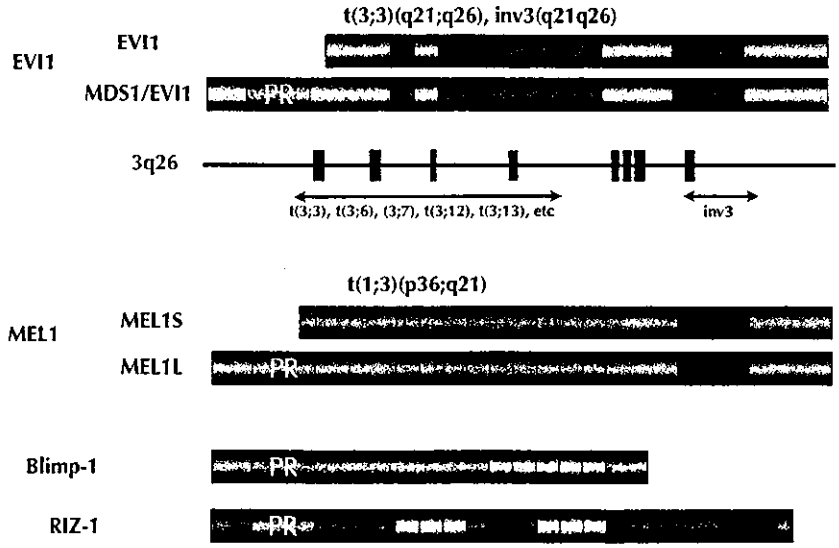

Figure 5. 1p36/3q26 Syndrome

small fusion products are translated. The *TEL* locus is involved in 12p deletions in MDS/AML, and the non-rearranged *TEL* allele seem to be inactivated in *TEL*-involving translocations[93]. These findings seem to support an idea that TEL acts as a tumor suppressor and that inactivation of TEL functions may lead to deregulated hematopoiesis and leukemogenesis.

***MLL* translocations**

MLL or *HTRX*, a human homologue of the *Drosophila* trithorax gene, is another target of balanced translocations. It was originally identified as a fusion partner of *AF4* from the 11q23 breakpoint of t(4;11)(q21;q23) translocation, which is closely associated with infantile biphenotypic leukemia[94]. Now an increasing number of fusion genes involving *MLL* have been identified from leukemia-associated translocations, including t(9;11)(q21;q23) and t(11;19)(q23;p13.3)[95]. In view of MDS pathogenesis, t(11;16)(q23;p13) and t(11;19)(q23;p13.1), as well as t(5;11)(q31;q23), have been reported in tMDS/tAML and molecularly delineated, in which CBP and MEN, as well as GRAF are fused with MLL, respecctively[96-98]. Tandem duplication of *MLL* has been also identified in some cases of MDS[99].

MLL is presumed to participate in epigenetic gene regulation that is relevant to development and differentiation of hematopoietic cells[100]. It interacts with SNF5, a component of a SWI/SNF complex[101] and contains a SET domain showing histone methyltransferase activity, which is lost in aberrant fusion proteins[102]. Thus MLL seems to regulate gene expression by chromatin modifications, and loss of its functions may be responsible for the pathogenesis of leukemia and MDS.

Translocations involving *NUP98* gene

11p15 is also a recurrent breakpoint found in tMDS/tAML, in which the *NUP98* gene is located. NUP98 is a nucleoporin involved in nuclear transport of protein and RNAs[103]. A number of chimeric proteins including NUP98/HOXA9, NUP98/DDX10, NUP98/HOXD13, NUP98/HOXB, NUP98/HOXC13, NUP98/TOP1 bave been reported in t(7;11)(p15;p15), inv(11)(p15q22), t(2;11)(q31;p15), t(11;17)(p15;q21), t(11;12)(p15;q13), and t(11;20)(p15;q11), respectively[104-108]. The other nucleoporin gene that participates in pathogenesis of AML/MDS is *CAN* or *Nup214*, which is fused with *DEK* to generate the *DEK/CAN* chimeric gene in t(6;9)(p23;q34), a translocation predicting a poor clinical outcome[109].

Unbalanced chromosomal abnormalities

7q-/-7 and der(1;7)(q10;p10) translocation

Monosomy 7 and a complete or partial loss of the long arm of chromosome 7 are of particular importance because these are among the most frequent cytogenetic lesions in MDS and associated with very poor prognosis[110-112]. In adults 7q-/-7 is usually seen in association with other cytogenetic abnormalities such as 5q-/-5[113], while it tends to be the sole abnormality in childhood. It may be found in *de novo* MDS (~20%), but more typically related to tMDS/tAML (~45%).

Monosomy 7 syndrome refers to a combination of monosomy 7 as the sole cytogenetic abnormality and development of myeloid neoplasms in childhood especially less than 4 years of age. Recurrent infections, hepatosplenomegaly, lymphadenopathy, defective neutrophil chemotaxis, a male predominance, and poor prognosis are among features that characterize this syndrome. Since monosomy 7 syndrome is prevalent in JMML cases, and since both monosomy 7 and JMML share many clinico-pathologic features in common, including frequent activation of the RAS pathway, there seems to be a significant overlapping between both entities[112]. Familial cases of monosomy7 and myeloid neoplasms are known, but in such cases monosomy 7 is not germline in origin, arguing that monosomy 7 is a consequence of some mutator effects from other genetic loci[114-117]. Based on chromosome banding analysis, two critical regions of 7q deletions have been delineated: one in 7q22 and the other in 7q32-q35[118-122], and detailed FISH-based analysis of 7q- has disclosed more heterogeneous groups of deletions. No TSGs have been successfully identified as a target of 7q-.

der(1;7)(q10;p10) is an unbalanced translocations found in ~2% of MDS and AML[61], especially of tMDS/tAML in association with use of alkylating agents[123-125]. Other clinical features of der(1;7)(q10;p10) include refractory cytopenia, trilineage displasia, a high propensity to leukemia, and a poor clinical outcome. Breakpoint analysis of der(1;7)(q10;p10) disclosed that the breakpoints are randomly distributed within the large clusters of centromere alphoid sequences (~0.5~3Mb), D1Z7 on chromosome 1 and D7Z1 on chromosome 7[126]. Thus no specific genes are involved in the breakpoints but loss of 7q and/or gain

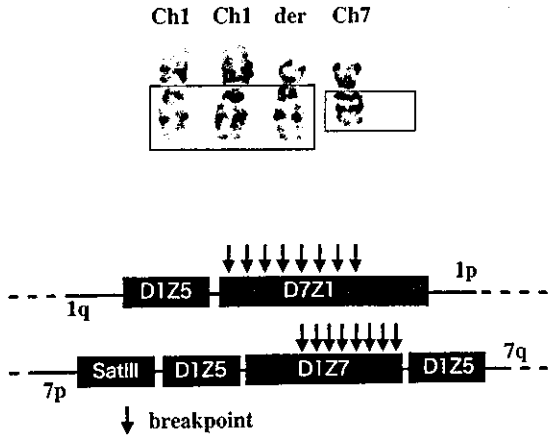

Figure 6. Partial karyotype of der(1;7)(q10;p10) and breakpoint distribution

of 1q materials resulting from the translocation should be important for the pathogenesis of MDS/AML having this translocation (Figure 6). Although it may represent a variant of 7q-, differences still exist in cytogenetic profiles between der(1;7)(q10;p10) and typical 7q-. der(1;7)(q10;p10) frequently occurs as the sole chromosomal abnormalities or in combination with one or two characteristic additional abnormalities, usually trisomy 8 and 20q-, while typical 7q- in adults usually appears as one of complex anomalies and in association with 5q-/-5, indicating der(1;7)(q10;p10) positive MDS/AML is likely to represent a distinct pathologic entity. In this regard, it is of note that 1q trisomy results from a number of similar 'centromeric unbalanced translocations' reported in MDS, including t(1;12)(q10;p10), t(1;15)(q10;p10), t(1;16)(q10;o10), t(1;18)(q10;p10), and t(1;21)(q10;p10), indicating roles of 1q+ in the pathogenesis of myeloid tumors[127].

5q-/-5

Abnormalities of chromosome 5 in MDS include monosomy 5, interstitial deletion of 5q and unbalanced translocations, among which interstitial deletion of 5q is most frequently observed. Especially, a combination of MDS and 5q- as the sole abnormality is referred to as 5q-syndrome and typically found in primary MDS cases[128]. It has a female predominance with a female to male ratio of 3:1 and generally shows rare leukemic transformation, very good prognosis, refractory anemia, high or normal platelet counts, and small hypolobulated megakaryocyts, which add up to a distinctive entity as found in the new WHO classification. On the other hand, 5q loss is also observed in combination with other cytogenetic abnormalities, such as 7q-/-7. Familial cases with 5q- and MDS have been also described[129].

Extent of 5q deletions is highly variable among different cases, but the critical region of 5q deletion seems to contain 5q31-q33. Of interest is that a number of cytokine-related genes, including genes for IL-3, IL-4, IL-5, M-CSF, GM-CSF, and M-CSF receptor, are clustered together and implicated in the pathogenesis of 5q-syndetome[130,131]. Other candidates of target genes for 5q deletion are *IRF-1*, *EGR-1*, and *PURA*-. IRF-1, a transactivator of interferon genes, is deleted in 90% of 5q- cases and thought to be a candidate for a relevant TSG for 5q-[132,133]. No tumor specific mutations, however, have thus far described for *IRF-1*[134]. In molecular cytogenetic approach, the smallest commonly deleted region has been currently narrowed to 1 to 1.5 Mb between D5S479 and D5S500[135].

Finally, with regard to the pathogenesis of 5q- syndrom, a recent report on a possible effectiveness of CC-5013 (REVIMID), a thalidomide analogue, may be intriguing, in which ten of eleven patients with 5q- syndrome achieved a complete transfusion independent response with REVIMID treatment and, very significantly, also resulted in disappearance of the chromosomal abnormality (5q-) in each of these patients (http://www.celgene.com/).

20q-

Deletion of 20q is most commonly found in MPD, particularly in polycythemia vera (~10%), but also described in ~5% of MDS and ~2% of AML[61]. 20q- appears solely or accompanied by other abnormalities such as 7q- and 5q-. It predicts generally favorable prognosis and, together with -Y and 5q-, is defined as a favorable cytogenetic abnormality in IPSS[136,137]. A previous report demonstrated that 20q- could not be detected in purified peripheral granulocytes in patients having 20q- in bone marrow cells despite that HUMARA assays unequivocally showed clonal granulopoiesis[138]. Thus it seems likely that 20q- may arise as a secondary event within the preexisting MDS clones, while 20q- positive clones may not contribute to mature granulopoiesis. The critical deletion spans from 20q11.2 to 20q13.2, which is now reduced to the regions between S20S17 and D20S174. Candidates of relevant TSGs include TOP1 and phospholipase C δ [139].

Other loss of chromosome materials

12p13 is another target of deletion not only for MDS and AML but also for lymphoid neplasms. The critical deletion was reportedly demarcated by *TEL* on the telomeric end and by *KIP1* on the centromeric end, and both genes are presumed to be candidates for the relevant TSGs of 12p deletion, as partly mentioned above[140]. KIP1 is a potent inhibitor of cyclin dependent kinases and takes a crucial role in cell cycle regulation[141]. No mutations have been detected in both *TEL* and *KIP1*, although *TEL* seems to be frequently inactivated by translocations. The short arm of chromosome 17 is also the target of deletion in MDS/AML and most frequently seen in tMDS/tAML cases (~6-10%). A presumptive target of this deletion is the *p53* gene, a well-established TSG. 13q deletion has been also recurrently described in MDS and involves regions between 13q14 and 13q21[142]. Within this interval, loss of the region covered by YAC 937C7, LSI/RB1, and YAC 745E3 appears to be a critical event in malignant myeloid cells[142]. This large region includes the smallest 13q segment lost in CLL, which is limited by *RB1* and the D13S25 marker. Loss of Y chromosome is found in MDS and AML (~8~10%) as the sole abnormality[61]. It occasionally occurs in healthy old men probably due to errors in cell division[143]. It may be postulated that loss of chromosome Y confers growth advantage and Y-missing progenitor cells acquire clonality during a long period of life, although most studies have denied involvement of Y-missing to leukemia development.

Trisomy 8

Trisomy 8 is the most frequent (~20-25%) numerical abnormality in AML and MDS, and more common in primary MDS as the sole abnormality[61,144]. It belongs to the intermediate-risk cytogenetic abnormality, while a recent report indicated a higher risk for leukemic transformation[145]. Although the relevant genes in +8 are mostly unknown, its role in leukemogenesis or MDS pathogenesis is inferred from rare cases with constitutional trisomy 8 mosaicism (CT8M), who present a high rate of developing different types of neoplasms especially of myeloid origins as well as other congenital abnormalities[146]. In some cases with MDS/AML, trisomy 8 may be derived from CT8M and possible manifestations of CT8M such as mental retardation should be carefully evaluated[147]. Acquired trisomy 8 seems to involve the CFU-GEMM population but to spare a pluripotent stem cell compartment and lymphoid lineages, suggesting a myeloid precursor origin of MDS or, alternatively, failure of +8-positive (sub)clones to contribute to lymphoid lineages[148].

Epigenetic abnormalities

In addition to genetic abnormalities, epigenetic alterations have been also implicated in the pathogenesis of MDS. A phenomenon that properties of cells are inherited to daughter cells by way of mechanisms other than primary sequences of genomic DNA is called epigenesis. Three mechanisms are known to mediate epigenetic processes in mammalian cells, DNA methylation, chromatin modifications, and genetic imprinting, among which DNA methylation has been most extensively studied in relation to human cancers[149].

Several TSGs, including the *p16INK4A*, *p15INK4B*, *VHL*, and *FHIT* genes, are frequently inactivated through hypermethylation of promoter sequences in many types of human cancers, and in this context, hypermethylation of *p15INK4B* has been best characterized in MDS. *p15INK4B* is an inhibitor of cyclin-dependent kinase (CDKs) strongly induced by TGF β stimulation and highly homologous to *p16INK4A*, which is one of the most frequently inactivated TSGs in human cancers[150,151]. In contrast to inactivation of *p16INK4A*, which is mostly caused by homozygous deletion in lymphoid malignancies[152], *p15INK4B* is inactivated in myeloid neoplasms exclusively through promoter hypermethylation[153-155]. Hypermethylation and inactivation of *p15INK4B* is much more frequent in high risk MDS (RAEB and RAEBt) (~50~80%) and AML derived from MDS (~100%) than low risk MDS(RA/RARS)[154], suggesting a possible importance of TGF β signaling in the pathogenesis of MDS in advanced stages.

Abnormal DNA methylation has been also implicated in MDS pathogenesis by its frequent response to demethylating agents, 5-aza-cytidine (Azacytidine) and 5-aza-2'-deoxycitidine (Decitabine)[156-158]. 5-aza-cytidine has been shown to ameliorate cytopenias and to prolong overall survival of high-risk MDS patients in a prospective randomized trial[156]. While demethylation of *p15INK4B* is observed after treatment with 5-aza-cytidine or 5-aza-2-deoxycitidine, other targets of abnormal methylation in MDS are currently unknown.

Conclusions

During the past two decades, a great deal of advance has

taken place in understandings of the molecular pathogenesis of MDS. A number of genetic abnormalities have been identified from analyses of characteristic balanced translocations in MDS/AML and of genes already shown to be mutated in other neoplastic diseases. On the other hand, however, many of these abnormalities are not specific to MDS or associated more with transformation to advanced stages than with *de novo* development of MDS, and we have little knowledge about genetic insults that initiate MDS. In view of clarifying the pathogenesis of early stages MDS, it is of crucial importance to identify molecular targets of chromosome deletions including 5q-/-5, 7q-/-7, and 20q-. In this regard, novel technologies have now become available that could facilitate identification of these targets, including high-density array-based comparative genomic hybridization (CGH) and high-throughput resequencing arrays[159,160]. Comprehensive analysis of gene expression profiling in MDS may also provide a valuable clue to this aim as well as to developing molecular diagnostics for MDS[161,162].

Furthermore, there exist other important aspects of MDS pathogenesis than genetic abnormalities, including immune-mediated mechanisms, stromal dysfunction, and abnormalities in angiogenesis (Figure 1). Immune-mediated mechanisms have been implicated in development of cytopenia especially in low-risk MDS. Oligoclonal T cell populations are frequently detected in the bone marrow from low risk MDS patients, which could disappear after treatment with immunosuppressive agents such as antithymocyte globulin[163,164] and cyclosporine A [165]. Possible involvement of autoimmunity is also inferred from the fact that the response of low-risk MDS to immunosuppressive therapy is closely related to a specific HLA subtype, HLA DRB1*1501 [166]. Although this review cannot afford to mention more details of these aspects, comprehensive understandings of MDS pathogenesis will clearly require full compilation of knowledge from the extending fields of research on this inexorable disorder.

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The EMBO Journal Vol. 21 No. 3 pp. 294–302, 2002

Integrity of intracellular domain of Notch ligand is indispensable for cleavage required for release of the Notch2 intracellular domain

Kiyoshi Shimizu[1,2], Shigeru Chiba[1,2], Toshiki Saito[1,2], Tokiharu Takahashi[1,2], Keiki Kumano[1,2], Yoshio Hamada[3] and Hisamaru Hirai[1,2,4]

Departments of [1]Hematology and Oncology and [2]Cell Therapy and Transplantation Medicine, Graduate School of Medicine, University of Tokyo, Tokyo and [3]National Institute of Basic Biology, Okazaki, Japan

[4]Corresponding author at: Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
e-mail: hhirai-tky@umin.ac.jp

The biological activity of the soluble form of the Notch ligand (sNL) and requirement of the intracellular domain (ICD) of the Notch ligand have been debated. Here we show that soluble Delta1 (sD1) activates Notch2 (N2), but much more weakly than full-length Delta1 (fD1). Furthermore, tracing the N2 molecule after sD1 stimulation revealed that sD1 has a defect in the cleavage releasing ICD of N2 (intracellular cleavage), although it triggers cleavage in the extracellular domain of N2. This represents the molecular basis of the lower activity of sD1 and suggests the presence of an unknown mechanism regulating activation of the intracellular cleavage. The fact that Delta1 lacking its ICD (D1Δ^{ICD}) exhibits the phenotype similar to that exhibited by sD1 indicates that the ICD of D1 (D1^{ICD}) is involved in such an as yet unknown mechanism. Furthermore, the findings that D1Δ^{ICD} acts in a dominant-negative fashion against fD1 and that the signal-transducing activity of sD1 is enhanced by antibody-mediated cross-linking suggest that the multimerization of Delta1 mediated by D1^{ICD} may be required for activation of the N2 intracellular cleavage.
Keywords: extracellular cleavage/intracellular cleavage/multimerization/Notch/Notch ligand

Introduction

The Notch family of genes encodes transmembrane receptors that are involved in the cell fate decision in vertebrates and invertebrates (Weinmaster, 1997; Greenwald, 1998; Artavanis-Tsakonas, 1999). In mammals, multiple Notch homologs have been identified, including Notch1 to Notch4 (Ellisen *et al.*, 1991; Weinmaster *et al.*, 1991, 1992; Kopan and Weitraud, 1993; Lardelli *et al.*, 1994; Uyttendaele *et al.*, 1996). The extracellular region comprises 29–36 epidermal growth factor (EGF)-like repeats and three copies of a Lin-12/Notch/Glp motif. The intracellular region contains cdc10/Ankyrin repeats and a PEST-containing domain. The Notch receptors are initially synthesized as ~300 kDa proteins, which are then proteolytically processed in the Golgi apparatus into an extracellular subunit (N^{EC}) containing multiple EGF repeats and lin-12/Notch repeats (Blaumueller *et al.*, 1997; Logeat *et al.*, 1998), and a single-pass transmembrane subunit (N^{TM}) containing a short extracellular tail and an intracellular domain (ICD; N^{ICD}). These subunits are reassembled in the *trans*-Golgi network and are presented as a heterodimeric, mature receptor at the cell surface (Blaumueller *et al.*, 1997). The lin-12/Notch repeats and Ca^{2+} ion are involved in maintaining the heterodimeric complex of N^{EC} and N^{TM} (Rand *et al.*, 2000).

Binding of a Notch ligand (NL) to N^{EC} triggers cleavage of Notch, releasing N^{ICD} from the cell membrane, which is then translocated into the nucleus to activate transcription of target genes in cooperation with RBP-Jκ (Kopan *et al.*, 1996; Chan and Jan, 1998; Jarriault *et al.*, 1998; Schroeter *et al.*, 1998; Struhl and Adachi, 1998; Shimizu *et al.*, 2000). This cleavage is mediated by a presenilin-containing complex and occurs within the transmembrane domain of Notch (intracellular cleavage) (De Strooper *et al.*, 1999; Struhl and Greenwald, 1999; Ye *et al.*, 1999). It has recently been proposed that prior to this cleavage, an additional cleavage at the extracellular domain of N^{TM} occurs in a ligand-dependent manner (Brou *et al.*, 2000; Mumm *et al.*, 2000) and that the extracellular cleavage autonomously promotes intracellular cleavage (Mumm *et al.*, 2000). However, these proposals were based on experiments using Notch1 (N1) proteins with most of the extracellular domain truncated, or experiments using a partial peptide of N1. Therefore, the relationships between ligand stimulation and cleavage of the extracellular region of the native Notch protein, and between ligand-induced extracellular cleavage and subsequent intracellular cleavage, have not been fully addressed.

Delta and Serrate (Jagged), comprising a Delta/Serrate/Lag-2 motif, tandem EGF repeats and a short ICD, are known to be ligands for the Notch receptor. As a natural protein *in vivo*, *Drosophila* Delta exists in both the transmembrane and soluble forms (Klueg *et al.*, 1998). It has recently been proposed that the soluble form of Delta is generated by Kuzbanian, a metalloprotease of the ADAM family (Qi *et al.*, 1999). Results of examinations of the biological activity of the soluble Notch ligands have been controversial. Whereas all experiments using cell-culture systems have shown that they behave as agonists (Li *et al.*, 1998; Qi *et al.*, 1999; Han *et al.*, 2000; Karanu *et al.*, 2000; Morrison *et al.*, 2000), *in vivo* experiments have demonstrated that soluble Delta and Serrate act as antagonists (Hukriede *et al.*, 1997; Sun and Artavanis-Tsakonas, 1997). It remains to be elucidated why such contradictory conclusions are drawn. To explain the discrepancy, the difference in the activity between the soluble and full-length forms should be clarified.

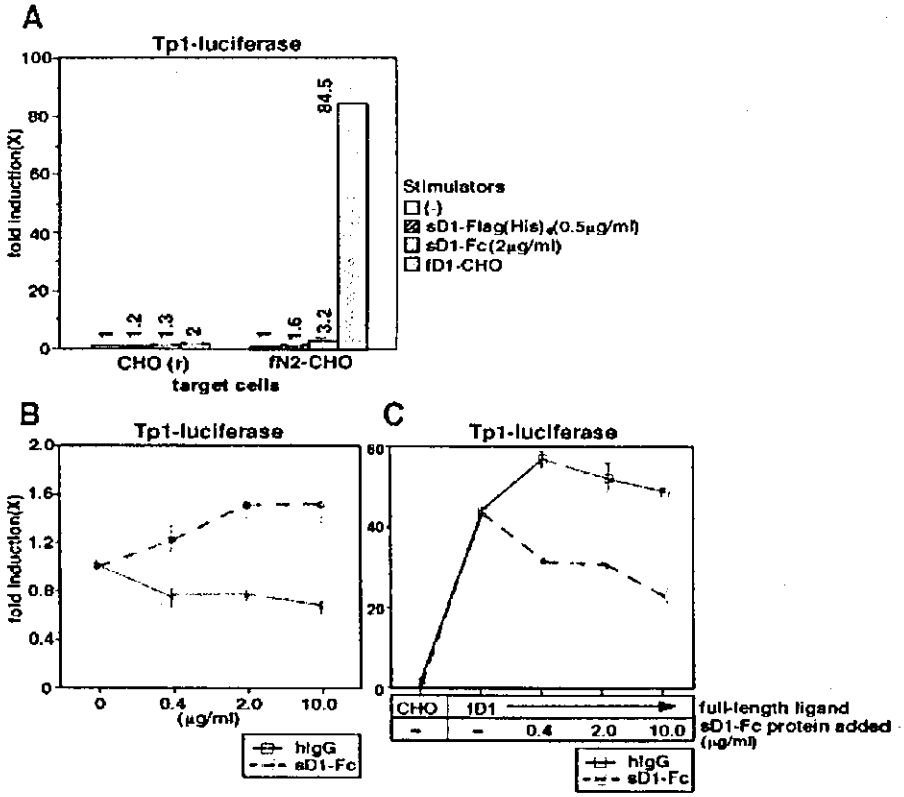

Fig. 1. Lower signal-transducing activity of soluble Delta1 protein. (A) Comparison of signal-transducing activity of sD1–Fc, sD1-Flag(His)$_6$ and fD1. A transient reporter assay with a TP1-luciferase reporter plasmid, pGa981-6, was performed using fN2-CHO cells. Following transfection of pGa981-6 into fN2-CHO, sD1–Fc, sD1-Flag(His)$_6$ or fD1-CHO was added to the transfected cells. Fold induction of the luciferase activity for each sample (mean of triplet measurements with standard deviation) was calculated against the control. The values are also shown in the graph. (B) N2-mediated transcriptional activation by sD1–Fc at increasing concentrations. Various concentrations of sD1–Fc were added to fN2-CHO cells transfected with pGa981-6. (C) The inhibitory effect of sD1–Fc on fD1-induced N2 signaling. fD1-CHO cells and sD1–Fc proteins at various concentrations were added simultaneously to the pGa981-6-transfected fN2-CHO cells. The same concentration of hIgG was added as a control.

In the present study, we show that the signal-transducing activity of the soluble form of Delta(-like-)1 (sD1) for Notch2 (N2) is obviously lower than that of full-length Delta1 (fD1), and that in coexistence with fD1 it inhibits the fD1-triggered N2 signal. This implies that sD1 is a partial agonist, while fD1 is a full agonist. Furthermore, we demonstrate the molecular basis of the impaired signal-transducing activity of sD1; it triggers cleavage of the extracellular domain of $N2^{TM}$, but promotes the cleavage step that releases $N2^{ICD}$ only very little. This indicates that, although the extracellular domain of NL alone is sufficient for extracellular cleavage, intracellular cleavage requires some other domain of NL, and that extracellular cleavage does not autonomously promote intracellular cleavage, suggesting the existence of an unknown mechanism that regulates the activation of the intracellular cleavage. Experiments using Delta1 without the ICD ($D1\Delta^{ICD}$) demonstrate that NL^{ICD} is important for the intracellular cleavage. Furthermore, the findings that $D1\Delta^{ICD}$ acts as a dominant-negative molecule against fD1 when they coexist and that the signal-transducing activity of sD1–Fc (sD1 fused to hIgG Fc portion) is enhanced by the addition of anti-Fc antibody suggest that oligomerization of NL is involved in Notch signaling.

Results

Lower signal-transducing activity of sD1

To define a biological activity of a soluble form of Notch ligand (sNL), we assessed the signal-transducing activity of mouse sD1 encompassing the entire extracellular region by comparing it with that of the full-length form in a transient reporter assay with CHO(r) cells overexpressing mouse full-length N2 (fN2-CHO), which is a highly sensitive assessment system for N2 signaling. Results showed that both Fc-fused and Flag(His)$_6$-tagged sD1 proteins [sD1–Fc and sD1-Flag(His)$_6$] activated the transcription of a reporter gene driven by the RBP-Jκ-responsive promoter, TP-1 (Figure 1A and B), but the transcriptional activity was obviously lower than that of fD1 [represented by the stimulation with CHO(r) expressing fD1 (fD1-CHO)] (Figure 1A). On the other hand, in coexistence with fD1, sD1 inhibited the fD1-mediated N2 activation compared with control hIgG (Figure 1C). These data indicate that sNL is a partial agonist, while full-length NL (fNL) is a full agonist.

We further evaluated the difference in the signal-transducing activity between the two molecules from another viewpoint, i.e. the nuclear accumulation of $N2^{ICD}$.

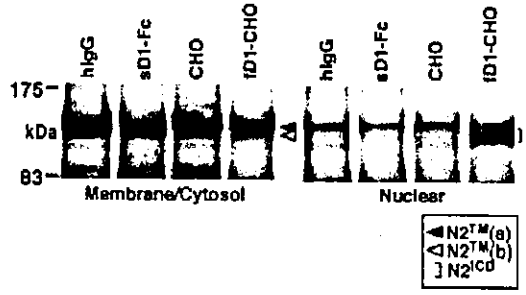

Fig. 2. N2 molecule traced after sD1–Fc and fD1 stimulations. BaF3 was stimulated for 1.5 h under the conditions indicated in the figure and then separated into membrane/cytosol-rich and nucleus-rich fractions. In each fraction, N2 fragments containing an ICD were analyzed by western blot analysis using the bhN6 antibody after immunoprecipitation with an anti-N2 polyclonal antibody.

It is generally accepted that the nuclear accumulation of N^{ICD} generated by cleavage within the transmembrane domain of the Notch receptor after fNL stimulation is associated with activation of the transcription of downstream genes in Notch signaling. To evaluate the cleavage and nuclear accumulation in a serial manner, we used BaF3 cells capable of displaying these two events following stimulation with fD1. As previously reported, stimulation with fD1 decreased the amount of $N2^{TM}$ in the membrane/cytosol fraction [designated $N2^{TM}$(a); Figure 2] and, instead, N2-derived fragments representing $N2^{ICD}$ were accumulated in the nuclear-rich fraction (Shimizu *et al.*, 2000). In contrast, the stimulation with sD1–Fc did not result in detectable $N2^{ICD}$ in the nuclear-rich fraction, although it also reduced the amount of $N2^{TM}$(a) in the membrane/cytosol fraction. Instead, a new band representing a protein smaller than $N2^{TM}$(a) emerged in the membrane/cytosol fraction [designated $N2^{TM}$(b); Figure 2]. The fact that hardly any $N2^{ICD}$ was generated after stimulation with sD1–Fc was compatible with the results of reporter assays (Figure 1).

sD1 has a defect in the cleavage required for release of $N2^{ICD}$, although it can trigger the extracellular cleavage of N2

To understand better the lower signal-transducing activity of sD1, we then characterized $N2^{TM}$(b) generated by sD1 stimulation, which was scarcely seen after fD1 stimulation (Figure 2). The decrease in the amount of $N2^{TM}$(a) and the appearance of $N2^{TM}$(b) in the membrane/cytosol fraction after stimulation with sD1–Fc (Figure 2) indicated that $N2^{TM}$(b) represented a molecule derived from $N2^{TM}$(a). A further fractionation of the membrane/cytosol fraction demonstrated that $N2^{TM}$(b) and $N2^{TM}$(a) were present in the membrane but not in the cytosol fraction (Figure 3A), suggesting that $N2^{TM}$(b) was a membrane-associated molecule lacking either the N- or the C-terminal tail of $N2^{TM}$(a). To determine which side of $N2^{TM}$(a) was cleaved to generate the $N2^{TM}$(b) fragment, we performed the two experiments. In the first, we used fN2-CHO(r), [CHO(r) with exogenous fN2 tagged with a Flag sequence at the C-terminus] to investigate whether the Flag tag remained in $N2^{TM}$(b) generated after sD1–Fc stimulation. The result was that the anti-Flag antibody detected $N2^{TM}$(b) (Figure 3B), indicating that $N2^{TM}$(b) lacks the N-terminus

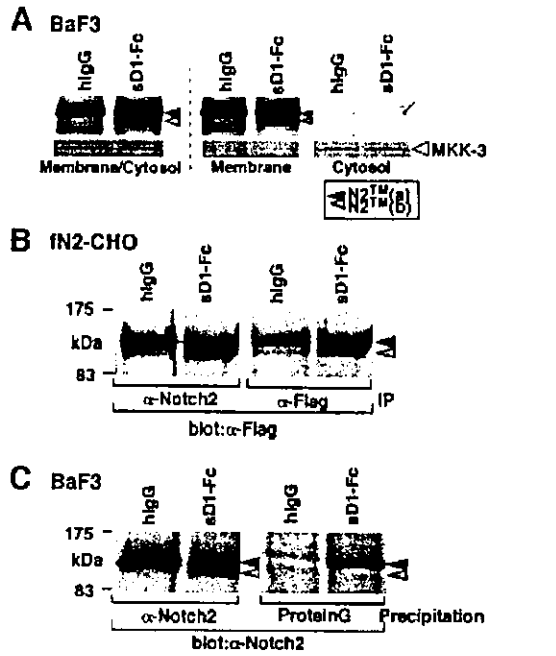

Fig. 3. Characterization of the $N2^{TM}$(b) fragment induced by sD1–Fc-stimulation. (A) To determine whether $N2^{TM}$(b) is a transmembrane protein, membrane/cytosol-rich fraction prepared from BaF3 after the sD1–Fc stimulation was then separated into membrane and cytosol fractions. N2 proteins in each fraction was subjected to western blot after immunoprecipitation with an anti-N2 polyclonal antibody. As a control for correct fractionation of membrane and cytosol franctions, an antibody against MKK3, MAP kinase, was used for each fraction in western blot analysis. (B) Generation of $N2^{TM}$(b) fragment containing a Flag$(His)_6$ tag at the C-terminus. fN2-CHO [CHO(r) with exogenous N2 with a Flag$(His)_6$ tag at the C-terminus] was incubated in the presence of either sD1–Fc or hIgG at 6.7 nM. After 1.5 h, the stimulated cells were collected and solubilized in a TNE buffer. The cell lysates were precipitated with an anti-Flag monoclonal (M2) or an anti-N2 polyclonal antibody. The precipitates were analyzed by western blot with the M2 antibody. IP, immunoprecipitation. (C) Co-precipitation analysis. BaF3 was incubated in RPMI medium containing sD1–Fc or hIgG at 6.7 nM for 1.5 h, then subjected to a cross-linking reaction to form the binding complex of sD1–Fc and N2. Following the reaction, the BaF3 lysates were divided into two aliquots. One was precipitated with an anti-N2 polyclonal antibody to identify N2 protein fragments. To precipitate sD1–Fc-containing complex, protein G beads were added directly to the other. These precipitates were analyzed by western blot with the bhN6 antibody.

but not the C-terminus of $N2^{TM}$(a). In the second experiment, we assessed whether $N2^{TM}$(b) was coprecipitated with sD1–Fc. In a previous report, we described that $N2^{TM}$(a) is precipitated with sD1–Fc (Shimizu *et al.*, 2000). If the cleavage after sD1 stimulation occurs within the short extracellular domain of $N2^{TM}$(a), sD1–Fc-bound $N2^{EC}$ probably loses the association with $N2^{TM}$(b), and thus $N2^{TM}$(b) is not coprecipitated with sD1–Fc. As expected, sD1–Fc coprecipitated only $N2^{TM}$(a) and not $N2^{TM}$(b) (Figure 3C). This result also suggests that $N2^{TM}$(b) was generated from $N2^{TM}$(a) by the cleavage in the juxtamembrane portion of the extracellular region (see Figure 7).

We then investigated whether the same cleavage occurred during the process of fD1-mediated N2 signaling, to verify that $N2^{TM}$(b) generated by sD1 was not an artifact. The amount of sD1–Fc binding to BaF3 was significantly reduced when the binding assay was performed after the co-culture of BaF3 with fD1-CHO, as

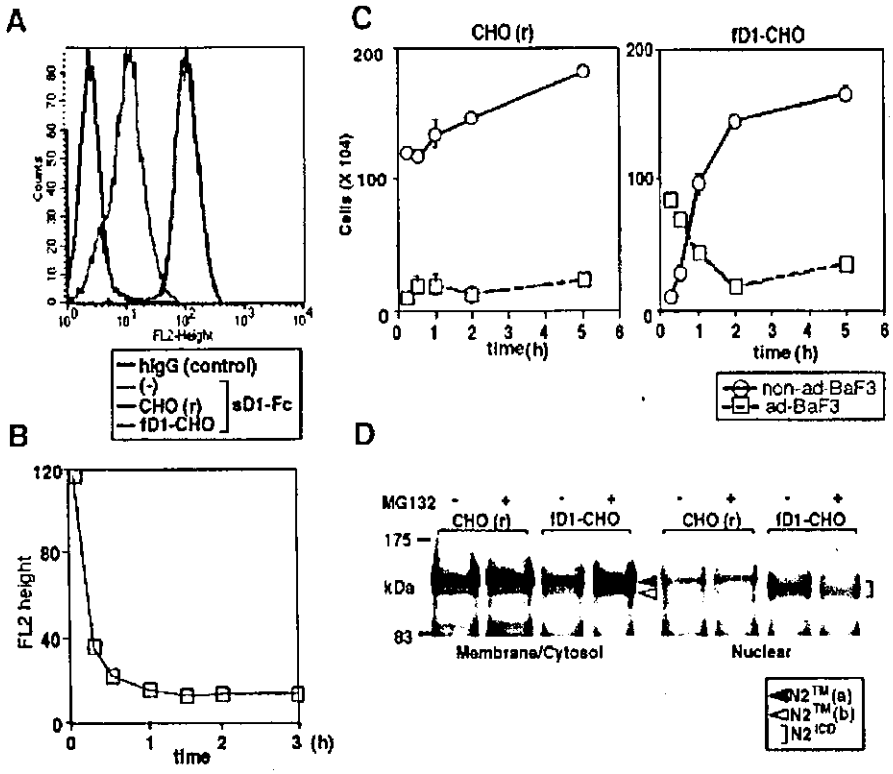

Fig. 4. Involvement of extracellular cleavage in fD1-mediated N2 activation. (**A**) Reduction in the amount of sD1–Fc binding to BaF3 after co-culture with fD1-CHO. BaF3 cells were collected at 1.5 h after co-culture with either CHO(r) or fD1-CHO. Cell-binding assay using sD1-Fc at 6.7 nM was performed for BaF3 cells recovered from the co-culture. (**B**) Time-course analysis of binding of sD1–Fc to BaF3 after co-culture with fD1-CHO. BaF3 cells co-cultured for the times indicated in the figure were subjected to cell-binding assays. The extent of fluorescence brightness giving the highest frequency (*y*-axis) was plotted against time (*x*-axis). (**C**) Time-dependent detachment of BaF3 from fD1-CHO. The time-course of the number of detached BaF3 cells was recorded in a cell–cell association assay. ad-BaF3, BaF3 that adhered to CHO cells; non-ad-BaF3, BaF3 that did not adhere to CHO cells. (**D**) Relationship between extracellular cleavage and nuclear transport of $N2^{ICD}$. MG-132, an inhibitor of cleavage for release of N^{ICD}, was added to a co-culture system of BaF3 and fD1-CHO at a final concentration of 25 μM. After 1.5 h of co-culture, the BaF3 cells were collected and separated into membrane/cytosol-rich and nucleus-rich fractions. In each fraction, N2 fragments containing an ICD were analyzed by western blot using the bhN6 antibody after immunoprecipitation.

compared with the co-culture with control CHO(r) (Figure 4A). A time-course analysis showed that the reduction in sD1–Fc binding started within 15 min and reached a plateau 1.5 h from the initiation of the co-culture (Figure 4B). During the co-culture, we observed that BaF3 cells, which previously adhered to fD1-CHO within 10 min, were detached from it in a time-dependent fashion (Figure 4C). One possible explanation for these phenomena is that the fD1-induced N2 extracellular cleavage results in the dissociation of $N2^{EC}$ together with the bound fD1 molecule from $N2^{TM}$, which then results in the reduction in fD1-bindable N2 receptors on BaF3 cell surface (see Figure 7).

To obtain more direct evidence of the extracellular cleavage of $N2^{TM}$(a) by fD1 and to determine the relationship between this extracellular cleavage and the cleavage following it, we added MG-132, a known inhibitor of the intracellular cleavage that results in the release of N^{ICD} (De Strooper *et al.*, 1999; Mumm *et al.*, 2000), into the co-culture system of BaF3 and fD1-CHO. The addition of MG-132 in fact reduced the amount of fD1-induced $N2^{ICD}$ in the nucleus-rich fraction (Figure 4D), implying that it prevented fD1-induced intracellular cleavage. In addition, $N2^{TM}$(b) was detected in the membrane/cytosol fraction when MG-132 was added (Figure 4D). This indicated that extracellular cleavage also occurred during the process of fD1-mediated N2 signaling, as in the case of sD1, and that stimulation with fD1 induced cleavage of $N2^{TM}$(a) in the extracellular region, prior to cleavage in the transmembrane region. The above findings lead to the conclusions that the extracellular cleavage does not autonomously trigger the N2 intracellular cleavage and that sD1 has a defect in the cleavage required for release of $N2^{ICD}$, although it can trigger extracellular cleavage of N2 (see Figure 7).

Requirement of NL^{ICD} for full activation of N2

To determine which region of Deltal is involved in progression of the intracellular cleavage, we generated a CHO(r) cell line expressing $D1\Delta^{ICD}$ ($D1\Delta^{ICD}$-CHO) and investigated its signal-transducing activity. Using cell-binding assays with sN1, we first confirmed that sN1 bound to fD1-CHO and $D1\Delta^{ICD}$-CHO in an indistinguishable manner (Figure 5A), indicating that fD1 and $D1\Delta^{ICD}$ were approximately equally expressed on the cell surface. We also observed that the amount of sD1–Fc binding to BaF3 after co-culture with $D1\Delta^{ICD}$-CHO was reduced to a degree similar to that after the co-culture with fD1-CHO

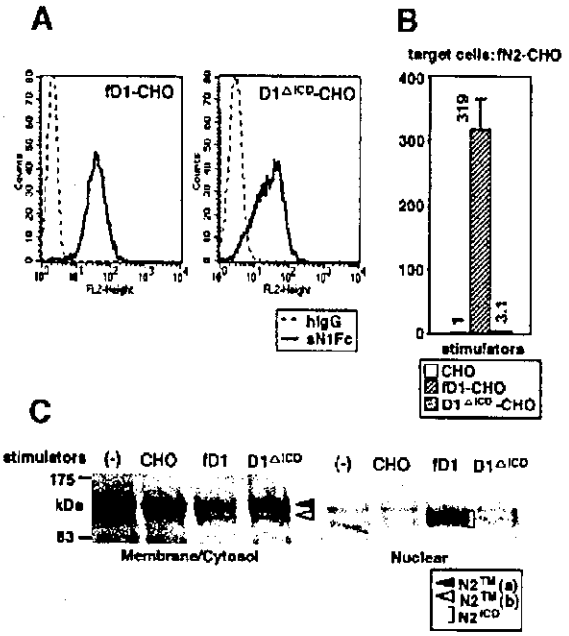

Fig. 5. Requirement of the intracellular domain of Delta1 for full activation of N2. (**A**) Generation of D1Δ^{ICD}-CHO [CHO(r) cells expressing the truncated Delta1 lacking its intracellular domain]. To investigate the expression of fD1 and D1Δ^{ICD}, a cell-binding assay using sN1-Fc (6.7 nM) was performed against the fD1-CHO and D1Δ^{ICD}-CHO cells. (**B**) Comparison of signal-transducing activity of fD1 and D1Δ^{ICD}. To examine activity of the two molecules, a transient reporter assay with a TP1-luciferase reporter plasmid was performed using fN2-CHO cells. Fold-induction of luciferase activity for fD1-CHO and D1Δ^{ICD}-CHO (mean of triplicate measurements with standard deviation) was calculated against luciferase activity when parental CHO(r) was used as stimulator. (**C**) N2 fragments after fD1 and D1Δ^{ICD} stimulations. BaF3 was stimulated for 1.5 h under the conditions indicated in the figure and then separated into two fractions, membrane/cytosol-rich and nucleus-rich. In each fraction, N2 fragments containing an intracellular domain were analyzed by western blot using the bhN6 antibody after immunoprecipitation.

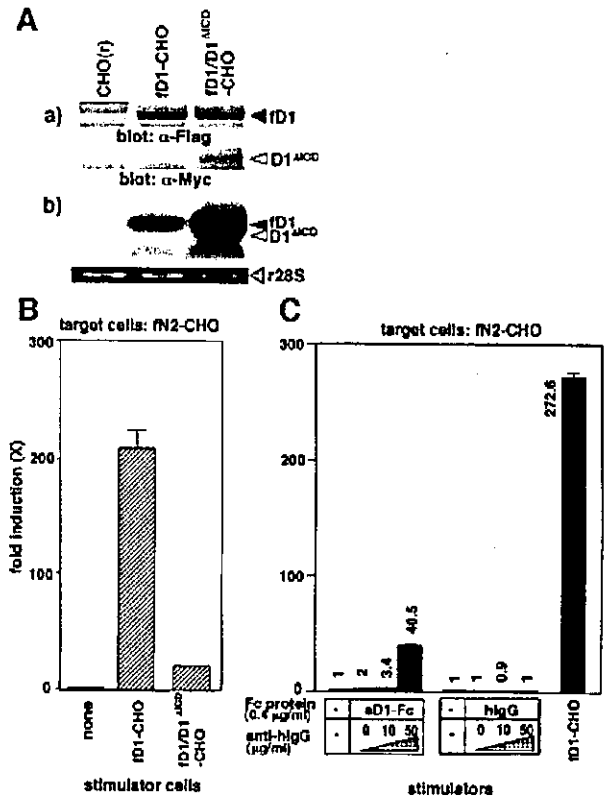

Fig. 6. Involvement of multimerization of Delta1 in the N2 activation. (**A**) Generation of fD1-CHO cells expressing Myc-tagged D1Δ^{ICD} (fD1/D1Δ^{ICD}-CHO). (a) Expression of Myc-tagged D1Δ^{ICD} and Flag-tagged fD1 proteins in fD1/D1Δ^{ICD}-CHO cells were examined by western blot analysis with an anti-Flag or an anti-Myc antibody. (b) To compare the expression levels of mRNA of fD1 with D1Δ^{ICD} in the fD1/D1Δ^{ICD}-CHO cells, total RNA (10 μg) extracted from the cells was subjected to northern blot using the 5′-end fragment of mouse *Delta1* cDNA as a probe. The lower panel shows ethidium bromide-stained 28S ribosomal RNA (r28S) in each lane. (**B**) Enhancement of the signal-transducing activity of sD1–Fc by addition of an anti-Fc antibody. A transient reporter assay was performed using pGa981-6 plasmid-transfected fN2-CHO cells in the presence of sD1–Fc and the anti-Fc antibody at various concentrations. hIgG was added as a control for sD1–Fc. The relative induction of luciferase activity in each sample (mean of triplicate measurements with standard deviation) was calculated against luciferase activity in the presence of hIgG alone. (**C**) A dominant-negative effect of D1Δ^{ICD} on fD1-triggered N2 activation. fD1/D1Δ^{ICD}-CHO [CHO(r) cells co-expressing fD1 and D1Δ^{ICD}] was generated and its signal-transducing activity was examined by a transient reporter assay with pGa981-6 plasmid-transfected fN2-CHO cells.

(data not shown), and that once D1Δ^{ICD}-CHO-adhered BaF3 cells were detached from it exactly like BaF3 cells co-cultured with fD1-CHO (data not shown). In contrast, the reporter assays using these cell lines showed that the signal-transducing activity of D1Δ^{ICD} was obviously lower than that of fD1 (Figure 5B). Correspondingly, N2^{ICD} was hardly detected in the nucleus-rich fraction after stimulation with D1Δ^{ICD}, unlike after stimulation with fD1, while D1Δ^{ICD} reduced the amount of N2^{TM}(a) in the membrane/cytosol fraction (Figure 5C). These observations indicate that D1Δ^{ICD} can bind to N2 and induce its extracellular cleavage, but cannot facilitate the ensuing intracellular cleavage, being similar to the phenotype exhibited by sD1, although emergence of N2^{TM}(b) was less clear when stimulated with D1Δ^{ICD} than that with sD1. Therefore, it was concluded that the ICD of D1 (D1^{ICD}) is essential for D1-induced N2 intracellular cleavage and full activation of N2, and that the lower signal-transducing activity of sD1 is a consequence of the lack of the ICD rather than the lack of the membrane anchorage.

Importance of multimerization of NL for full activation of N2

To see an effect of D1Δ^{ICD} on fD1-triggered N2 activation in the coexistence of the two molecules, we generated the fD1-CHO cell line expressing D1Δ^{ICD} (fD1/D1Δ^{ICD}-CHO) (Figure 6A) and investigated its signal-transducing activity. The result showed that the intensity of the N2 signal transduction by fD1/D1Δ^{ICD}-CHO was about one-tenth of that by fD1-CHO, indicating that the activity of fD1 was reduced to about one-tenth in the presence of D1Δ^{ICD} (Figure 6B). This suggests that D1Δ^{ICD} acts in a dominant-negative fashion against fD1, in agreement with previous report indicating that the Delta proteins lacking the ICD act as dominant-negative proteins in *Drosophila* and vertebrates (Chitnis *et al.*, 1995; Sun and Artavanis-Tsakonas, 1996; Jen *et al.*, 1997). Since the expression level of D1Δ^{ICD} was less than that of fD1 in the fD1/ D1Δ^{ICD}-CHO cells [Figure 6A, (b)], the strong