J. Biochem. 128, 399–405 (2000)

A Subunit of the Mammalian Oligosaccharyltransferase, DAD1, Interacts with Mcl-1, One of the bcl-2 Protein Family[1]

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Received May 1, 2000; accepted June 15, 2000

DAD1 is a mammalian homologue of *Saccharomyces cerevisiae* Ost2p, a subunit of the oligosaccharyltransferase complex. Loss of its function induces apoptosis in hamster BHK21 cells. By means of a two-hybrid method involving DAD1 as bait, the C-terminal region of Mcl-1, one of the bcl-2 family, was isolated. Consistently, DAD1 binds well to Mcl-1 in COS cells when overexpressed. On deletion analysis, the C-terminal domain of Mcl-1 containing BH[2] (bcl-2 homologous domain) was found to be essential for the interaction with DAD1. On the other hand, the C-terminal half of DAD1 was concluded to be essential for the interaction with Mcl-1. Surprisingly, a ΔC-DAD1 mutant lacking only 4 amino acid residues from the C-terminus did not complement the tsBN7 mutation, while it interacted well with Mcl-1. In contrast, ΔN-DAD1 lacking 20 amino acid residues from the N-terminus still exhibited the ability to complement the tsBN7 mutation. Thus, the C-terminus of DAD1 was suggested to play an important role in *N*-linked glycosylation and to complement the tsBN7 mutation. Mcl-1 may be required for the inhibition of apoptotic cell death caused by a loss of DAD1.

Key words: apoptosis, DAD1, Mcl-1, *N*-linked glycosylation, tsBN7.

Apoptosis is a genetically determined suicide-program which eliminates extra cells within a multicellular organism during cellular differentiation and development (*1*). Although there has been a great progress in the determination of factors that participate in this program, the details have yet to be clarified. In mammals, the homologues of ced-3 are well known as a growing family of cystein proteases called caspases, whereas the homologues of ced-9 belong to the bcl-2 family of proteins (*2*). The bcl-2 family consists of two types of proteins that have distinct roles (*3*). One of them prevents the programmed cell-death, and the other comprises inducers of cell death. The Mcl-1 protein belongs to the bcl-2 family, which is known to prevent cell death, and is essential for animal development (*4*). It was first cloned as a protein expressed in myeloid cell differentiation (*5*). Although Mcl-1 functions in a similar way to bcl-2, the intracellular distribution of Mcl-1 is somewhat different from that of bcl-2. Mcl-1 proteins exist mainly in the mitochondria, but a large population also exists in the light membrane fraction, in which the endoplasmic reticulum is found (*6*). Mcl-1 inhibits the apoptosis induced by c-myc in Chinese hamster ovary cells (*7*), or delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions (*8*). It dimerizes with another member of the bcl-2 protein family, Bax, but does not for a dimer by itself, nor does it dimerize with bcl-2 (*9*).

DAD1 (Defender of Apoptotic cell Death) was identified as the mutated gene in the tsBN7 cell line, a temperature-sensitive (ts) growth-mutant of the hamster BHK21 cell line (*10*, *11*). At a nonpermissive temperature, DAD1 proteins are lost due to the mutation in tsBN7 cells, causing apoptosis. Hence, DAD1 was thought to be a negative regulator of apoptosis, while it does not exhibit any sequence similarity to other genes related to apoptosis. DAD1 has been highly conserved throughout evolution. The plant DAD1 gene rescues the ts growth-defect of the tsBN7 cell line (*12*, *13*). The *Saccharomyces cerevisiae* DAD1 homologue is OST2, which is one of the subunits comprising the oligosaccharyltransferase complex (*14*). While overproduction of Ost2p does not rescue the ts growth of tsBN7 cells, the *N*-linked glycosylation is defective in tsBN7 cells (*15*), therefore there is some functional relationship between DAD1 and Ost2p. It could be possible that tsBN7 cells enter apoptosis due to a defect in the *N*-linked glycosylation. Consistently, tunicamycin, an inhibitor of protein *N*-glycosylation, induces apoptosis in human HL-60 cells (*16*). Such induction of apoptosis could be negatively regulated by Mcl-1, to which DAD1 tightly binds.

MATERIALS AND METHODS

Cell Culture and Antibodies—BHK21, tsBN7, and COS-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, streptomycin, and kanamycin. Antibodies were as follows; anti Mcl-1 polyclonal antibodies (Pharmingen), anti T7-tag monoclonal antibodies (Novagen), anti-HA monoclonal antibody, and the anti DAD1 polyclonal antibodies described previously (*15*). tsBN7, BHK21, and COS-7 cells were transfected by lipofection using LIPOFECTAMINE (Gibco BRL) following

[1] This work was supported by Grants-in-Aid for Specially Promoted Research from the Ministry of Education, Science, Sports and Culture of Japan.

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the manufacturer's instructions.

Plasmid Construction—pcDEBT7-Mcl-1: Mcl-1 cDNA was amplified by PCR using primers 5′-GGT GTG AAT TCA TGT TTG GCC TCA AAA G-3′ and 5′-CGG GAA TTC CTA TCT TAT TAG ATA TGC C-3′ from the human B cell library in pACT (by S. Elledge) as the template. The resulting PCR products were either subcloned into the PET28 vector (Novagen), resulting in T7-fused *Mcl-1*, which was then subcloned into the mammalian expression vector pcDEB.

Deletion of Mcl-1 cDNA: The ΔN-Mcl-1 cDNA encoding amino acid residues 135–350 of Mcl-1 was amplified by PCR using primers 5′-GAA TTC GAG CCT CTC GGG AAG CGG-3′ and 5′-CGG GAA TTC CTA TCT TAT TAG ATA TGC C-3′. The ΔTM-Mcl-1 cDNA encoding amino acids 1–330 of Mcl-1 was amplified by PCR using primers 5′-GGT GTG AAT TCA TGT TTG GCC TCA AAA G-3′ and 5′-CAT TCC TGA TGC AAG CTT ATA GGT CCT CTA C-3′. The ΔBH^2-Mcl-1 cDNA encoding amino acids 1–305 of Mcl-1 was amplified by PCR using primers 5′-GGT GTG AAT TCA TGT TTG GCC TCA AAA G-3′ and 5′-GGG AAT TCT TAT GTC CTT ACG AG-3′. The ΔBH^3-Mcl-1 cDNA encoding amino acids 226 to 350 of Mcl-1 was amplified by PCR using primers 5′-GGG AAT TCG AGA CGG TCT TCC AAG G-3′ and 5′-CGG GAA TTC CTA TCT TAT TAG ATA TGC C-3′. The BH^{4+3}-Mcl-1 cDNA encoding amino acids 1–225 of Mcl-1 was amplified by PCR using primers 5′-GGT GTG AAT TCA TGT TTG GCC TCA AAA G-3′ and 5′-GGG AAT TCC TAG TGG TTG CGC TG-3′. The BH^4-Mcl-1 cDNA encoding amino acids 1–131 of Mcl-1 was amplified by PCR using primers 5′-GGT GTG AAT TCA TGT TTG GCC TCA AAA G-3′ and 5′-GGG AAT TCC TAG TAC CCG TCC AG-3′. The BH^{1+2}-Mcl-1 cDNA encoding amino acids 240-327 of Mcl-1 was amplified by PCR using primers 5′-GGG AAT TCG ACG ATG TGA AAT CG-3′ and 5′-GGG AAT TCG CCA CCT TCT AGG TC-3′. The ΔBH^1-Mcl-1 cDNA encoding amino acids 283–350 of Mcl-1 was amplified by PCR using primers 5′-GGG AAT TCC AAG AAA GCT GCA TC-3′ and 5′-CGG GAA TTC CTA TCT TAT TAG ATA TGC C-3′. A T7-tag was fused to the N-terminus of the resulting PCR products, followed by subcloning into the mammalian expression vector pcDEB.

Deletion of DAD1: The following regions of DAD1 were amplified by PCR using the indicated primers: ΔNDAD1 (21–113 a.a.), Primers 5′-GGT CTA GAA TGG GAT ACC CAT ACG ATG TTC CAG ATT ACG CTC CGC AGC GGC TGA AGT TGC-3′ and 5′-CCA AGC TTT CAG CCA ACG AAG TTC ATG AC-3′ (primer 5); ΔCDAD1 (1–109 a.a.); primers 5′-GGT CTA GAA TGG GAT ACC CAT ACG ATG TTC CAG ATT ACG CTA TGT CGG CGT CTG TGG-3′ (primer 1) and 5′-CCA AGC TTT CAC ATG ACG ACA AGG TGC AG-3′; Δ3DAD1 (1–78 a.a.), primers 1 and 5′-CCA AGC TTT CAG TTG ATC TGT ATT CTC AGG-3′; Δ1DAD1 (59–113 a.a.), primers 5′-GGT CTA GAA TGG GAT ACC CAT ACG ATG TTC CAG ATT ACG CTG GCT TCA TCT CTT GTG TGG-3′ and primer 5. The resulting PCR products, with a HA-tag fused to their N-termini, were integrated into the pcDEB vector, resulting in pcDEBHA-DAD1 clones.

Immunoprecipitation and Immunoblotting—BHK21 or COS cells were collected 48 h after transfection, washed with PBS, and then lysed by rotation at 4°C for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 0.5% NP-40). The cell lysates were incubated with 1–5 mg of the antibodies for 2 h and then incubated with either protein-G Sepharose beads (Pharmacia Biotech) or protein-A Sepharose beads (Sigma) for 30 min at 4°C. The beads were washed three times with the lysis buffer, resuspended in the SDS sample buffer, and then analyzed by SDS-PAGE for immunoblotting using the antibodies indicated. Immunoblotting was carried out as described previously (*15*), except that 11.25% polyacrylamide gels were used for detecting Mcl-1 constructs, and 15 or 10–20% gradient gels were used for detecting DAD1 constructs.

Two-Hybrid Screening and β-Galactosidase Activity Assay—S. cerevisiae strain Y190 was transformed with pAS-DAD1, which contains a DAD1 cDNA fused with the GAL4 DNA-binding domain, and was selected on a synthetic medium plate lacking tryptophan (*trp*⁻). The resulting transformants were cultivated in 50 ml of *trp*⁻ synthetic medium for one day, transferred to 500 ml of YPD medium and then cultivated for another 4 h. Cells were harvested, washed with sterile water and then treated with LiCl to be transformed by a human B cell library fused with the GAL4 activation-domain of the pACT vector (a gift from Dr. S. Elledge). Transformants were cultured in synthetic medium lacking leucine and tryptophan (*leu*⁻, *trp*⁻) overnight, and then spread on selection plates lacking histidine, leucine, and tryptophan (*his*⁻, *leu*⁻, *trp*⁻), but containing 25 mM 3-aminotriazole (3-AT). For the β-galactosidase assay, isolated colonies were grown in *leu*⁻, *trp*⁻ synthetic medium containing raffinose instead of glucose. One unit was taken as 1 nmol *o*-nitrophenyl-β-D-galactoside cleaved per min per mg protein at 28°C.

RESULTS

Isolation of Proteins Interacting with DAD1—In order to determine the functions of DAD1, proteins interacting with DAD1 were screened by means of a two-hybrid method with DAD1 as the bait. pAS-DAD1 carrying a DAD1 cDNA fused with the GAL4 DNA binding domain was constructed and transfected into strain Y190. A human B cell library fused with the GAL4-activation domain of the pACT vector (a gift from Dr. S. Elledge) was introduced into the resulting Y190 transfectants. Transfected cells were cultured overnight in synthetic medium lacking leucine and tryptophan, and then spread on selection plates lacking histidine, leucine, and tryptophan, but containing 25 mM 3-AT. Out of the 1.1×10^6 colonies screened, 25 were found to be his⁻ and 3-AT positive. Ost2p, the *S. cerevisiae* DAD1 homologue, comprises an oligosaccharyltransferase complex (*14*). In this regard, proteins required for the enzyme activity of oligosaccharyltransferase were expected to be isolated. However, no cDNA clones encoding a subunit of oligosaccharyltransferase were isolated. Very interestingly, however, one of the cDNA clones encoded the C-terminus region of the well known bcl-2 homologue Mcl-1 (*5*) (Fig. 1). As shown in Table I, the ability of DAD1 to interact with the C-terminal region of Mcl-1 is comparable to that of a small GTPase Ran to interact with RanBP2, a well known protein–protein interaction (*17*). The other cDNA encoded Nip3, which is also involved in apoptosis (*18*). These results suggested that DAD1 is involved in the programmed cell death causing apoptosis in addition to *N*-linked glycosylation. To address this issue, we investigated the relationship

between DAD1 and Mcl-1.

Mcl-1 and DAD1 Bind In Vivo—Although ΔN-Mcl-1, a C-terminal region of Mcl-1, strongly binds to DAD1, no interaction was observed between DAD1 and the full length Mcl-1 protein with the two-hybrid method (Fig. 1A and Table I). This may indicate that the observed interaction between DAD1 and Mcl-1 is artificial. In order to confirm the interaction between DAD1 and Mcl-1 *in vivo*, T7-tagged Mcl-1 cDNA was constructed and co-expressed with HA-tagged DAD1 in COS cells as described (*15*). Cultures of COS cells transfected with pcDEBT7Mcl-1 and pcDLSRa-HADAD1 were incubated for 48 h at 37.5°C and then lysed to carry out immunoprecipitation with the monoclonal antibodies (mAb) to either the T7- or HA-tag. Proteins immunoprecipitated with the mAB were analyzed by immunoblotting with the mAb to either the HA- or T7-tag, respectively (Fig. 2). When T7-tagged Mcl-1 was immunoprecipitated, a considerable amount of HA-fused DAD1 was co-imunoprecipitated (Fig. 2, lane 2). On the other hand, a little but significant amount of T7-tagged Mcl-1 was co-immunoprecipitated with HA-fused DAD1 (Fig. 2, lane 8).

DAD1 Binds to the C-Terminal Region of Mcl-1—We next determined which region of the Mcl-1 protein is important for binding with DAD1. As shown in Fig. 3A, Mcl-1 possesses four BH (bcl-2 homology) domains, and, at the C-terminal end, a transmembrane domain (TM) (*5*). A series of deleted Mcl-1 cDNA clones, which were fused in frame with the T7-tag at the N-terminus, were constructed and co-expressed with the HA-tagged DAD1 cDNA in COS cells. While HA-fused DAD1 was well expressed, some of the deleted Mcl-1 proteins; ΔBH^3, ΔBH^1, ΔBH^2, and ΔBH^{1+2}, were poorly expressed (Fig. 3B). However, when immunoprecipitated, these proteins were clearly each detected as a band corresponding to the expected molecular mass on immunoblotting with the mAb to the T7-tag (Fig. 3B, arrowheads), indicating that all the deleted Mcl-1 proteins were expressed. It is noticeable that the amount of DAD1 proteins co-immunoprecipitated with ΔBH^3-Mcl-1 was comparable to that of DAD1 precipitated with the full length Mcl-1. On the other hand, HA-DAD1 was not co-immunoprecipitated with BH^4 and BH^{4+3}, both of which were well expressed (Fig. 3B, lanes 5 and 6), revealing that the region from 1 to 225 a.a. of Mcl-1 is not required for binding to DAD1. Upon deletion of the transmembrane domain, the ability of Mcl-1 to bind DAD1 was significantly reduced. Even so, HA-DAD1 was apparently coimmunoprecipitated with ΔBH^2, ΔTM, and BH^{1+2}, all of which lack the transmembrane domain (TM). Taking these results together, the C-terminal region (283 to 327 a.a.) of Mcl-1 was concluded to be essential for the interaction between DAD1 and Mcl-1, which possesses a BH^2 domain.

The C-Terminal Region of DAD1 Is Required for Binding to Mcl-1—In order to further confirm the interaction between DAD1 and Mcl-1, the domain of DAD1 required for binding to Mcl-1 was determined. A series of deletions were introduced into DAD1 cDNA from either the C-terminus or the N-terminus, and the HA-tag was fused in frame to the N-terminus of the deleted DAD1 cDNAs as described under "MATERIALS AND METHODS" (Fig. 4A). The resulting cDNA clones were co-introduced into COS cells with T7-tagged Mcl-1. After 48 h incubation, total cell lysates were prepared and subjected to immunoblotting analysis with the mAb to either the T7- or HA-tag. As shown in Fig. 4B, T7-tagged Mcl-1 was well expressed, but the deleted DAD1 proteins were poorly expressed except for full length DAD1 (Fig. 4B, total cell lysate; anti-T7 and anti-HA). Both ΔC-DAD1 encoding the region from 1 to 109 a.a. and Δ1-DAD1 encoding the region from 59 to 113 a.a. were apparently co-immunoprecipitated with T7-Mcl-1 (Fig. 4B, IP:T7 tag, compare lanes 2 and 5). Consistently, when the deleted DAD1 proteins were immunoprecipitated with the mAb to HA, T7-Mcl-1 proteins were co-immunoprecipitated with ΔC-DAD1 and Δ1-DAD1 (Fig. 4B, IP:HA, lanes 2 and 5). Therefore, the region of DAD1 from 59 to 109 a.a. was concluded to be essential for binding to Mcl-1.

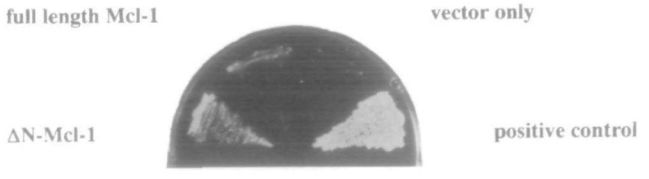

Fig. 1. **Interaction between DAD1 and Mcl-1.** *S. cerevisiae* strain Y190 carrying pAS-DAD1 was transformed with plasmids carrying the indicated cDNAs; full length Mcl-1, ΔN-Mcl-1, and pAS vector alone, and grown on a synthetic medium (*leu*$^-$, *trp*$^-$, *his*$^-$, 25 mM 3AT) plate. As a positive control, *S. cerevisiae* strain Y190 carrying pAS-RAN was transfected with pACT-RanBP2 fragment G5 (*17*).

TABLE I. **Interaction of DAD1 with Mcl-1: quantitative assaying of β-galactosidase activity from the *lacZ* reporter construct.**

Plasmids co-transfected		β-Galactosidase activity (units)
pASI	pACT II	
DAD1	Full length Mcl-1	0.69
DAD1	ΔN-Mcl-1	3.03
Ran	RanBP2	4.50
DAD1	Vector only	0.55

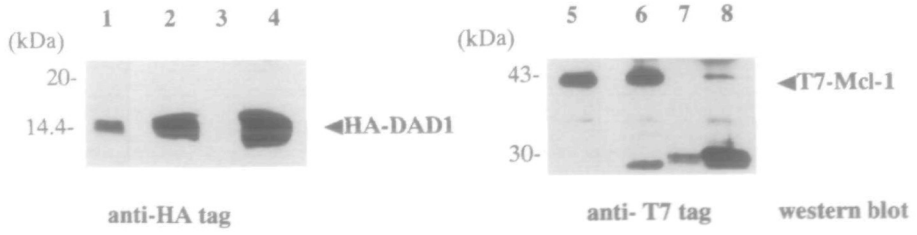

Fig. 2. **Mcl-1 is co-immunoprecipitated with DAD1.** 2×10^6 COS-7 cells were co-transfected with HA-tagged DAD1 and T7-tagged Mcl-1 cDNAs as described under "MATERIALS AND METHODS." After 48 h, transfected cells were collected, lysed and then subjected to immunoprecipitation with the mAb to the T7- (lanes 2 and 6) or HA- (lanes 4 and 8) tag. The precipitated samples were analyzed by immunoblotting using the mAb to the HA-tag (lanes 1 to 4) or T7-tag (lanes 5 to 8). Lanes 1 and 5: total lysates. Lanes 3 and 7: cell lysates immunoprecipitated with the pre-immune serum. Arrowheads indicate the positions of HA-DAD1 and T7-Mcl-1, respectively.

In order to examine the biological significance of the interaction between DAD1 and Mcl-1, a series of deleted DAD1 cDNA clones carried on the pcDEB vector, which possesses the hygromycin B resistance (HBr) gene, was introduced into tsBN7 cells. Transfected cells were incubated at either 33.5°C in the presence of hygromycin B or 39.5°C, a nonpermissive temperature for tsBN7 cells, to determine the ability of the deleted DAD1 proteins to complement the tsBN7 mutation. As shown in Table II, at a permissive temperature, the number of hygromycin B–resistant colonies was comparable for all the transfected cells, indicating that the transfection efficiency was comparable in all cases. On the other hand, at 39.5°C, cells transfected with the full-length DAD1 or ΔN-DAD1 lacking the N-terminal 20 amino acid residues grew to form colonies. Surprisingly, ΔC-DAD1 (encoding the region from 1 to 109 a.a.), which lacks only 4 amino acid residues from the C-terminus, did not confer the ts^+ phenotype to tsBN7 cells, although it bound well to Mcl-1. Compared to ΔN-DAD1 proteins which were not detected on immunoblotting (Fig. 4B, lane 4, anti-HA), ΔC-DAD1 cDNAs were significantly expressed in COS cells (Fig. 4B, lanes 2). The inability of ΔC-DAD1 to complement the tsBN7 mutation, therefore, could not be due to poor expression of ΔC-DAD1 cDNA.

DAD1 and Mcl-1 Are Partly Colocalized—The above results indicated that DAD1 bound to Mcl-1. Previously we found that DAD1 is localized in the ER (*15*). In order to examine the cellular localization of Mcl-1, wild-type Mcl-1 and T7-tagged DAD1 cDNAs were co-introduced into COS-7 cells. After 48 h incubation, cells were fixed and stained

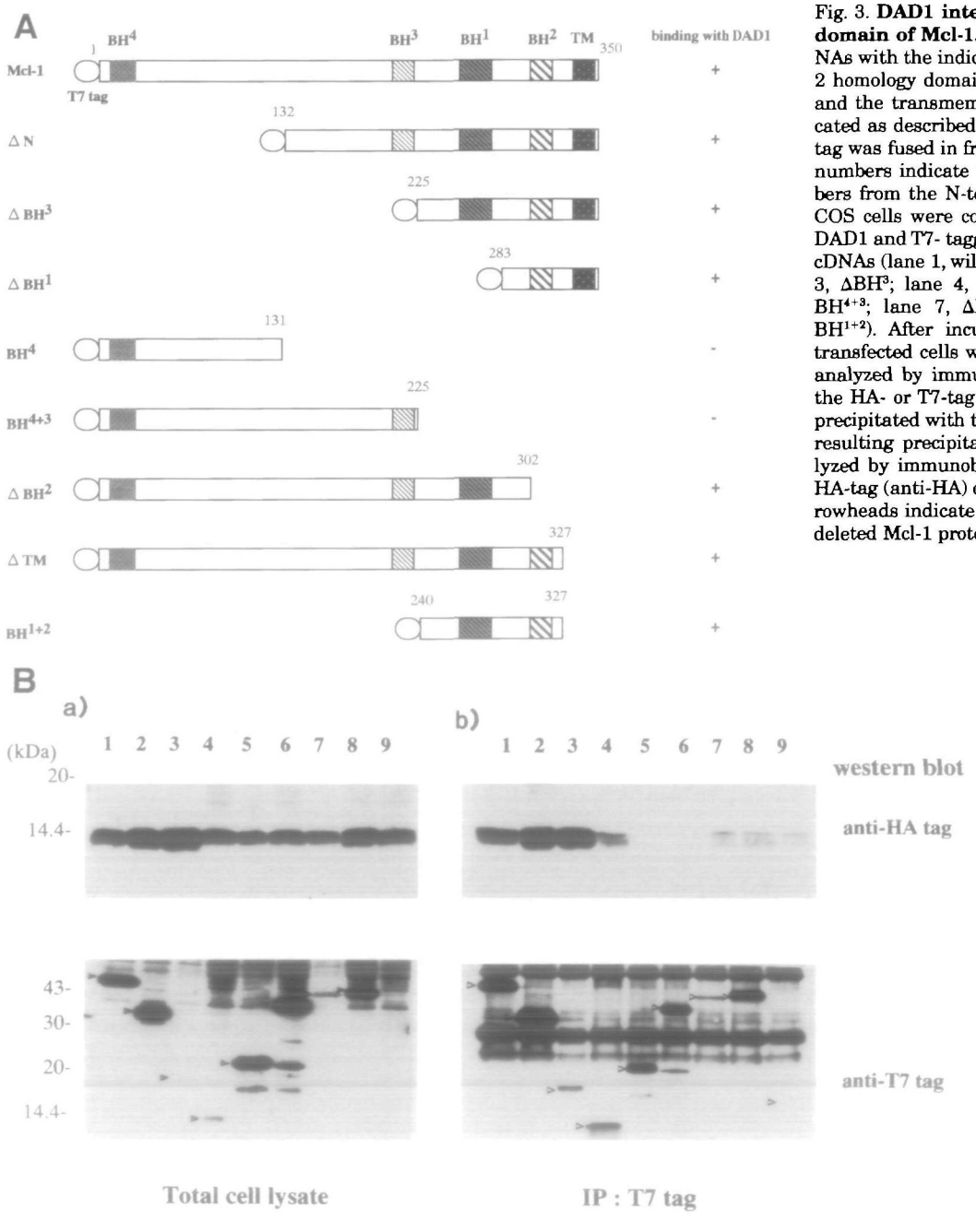

Fig. 3. **DAD1 interacts with the C-terminal domain of Mcl-1.** A: Construction of Mcl-1 cDNAs with the indicated regions deleted. The bcl-2 homology domains, BH^1, BH^2, BH^3, and BH^4, and the transmembrane domain, TM, are indicated as described (*5*). At the N-terminus, a T7-tag was fused in frame to the Mcl-1 cDNAs. The numbers indicate the amino acid residue numbers from the N-terminus of Mcl-1. B: 2×10^6 COS cells were co-transfected with HA-tagged DAD1 and T7- tagged Mcl-1 wild type or deleted cDNAs (lane 1, wild-type Mcl-1; lane 2, ΔN; lane 3, ΔBH^3; lane 4, ΔBH^1; lane 5, BH^4; lane 6, BH^{4+3}; lane 7, ΔBH^2; lane 8, ΔTM; lane 9, BH^{1+2}). After incubation at 37.5°C for 48 h, transfected cells were collected, lysed and then analyzed by immunoblotting with the mAb to the HA- or T7-tag as indicated (a), or immunoprecipitated with the mAb to the T7-tag (b). The resulting precipitated samples were then analyzed by immunoblotting with the mAb to the HA-tag (anti-HA) or T7-tag (ant-T7 tag). The arrowheads indicate the position of wild-type and deleted Mcl-1 proteins.

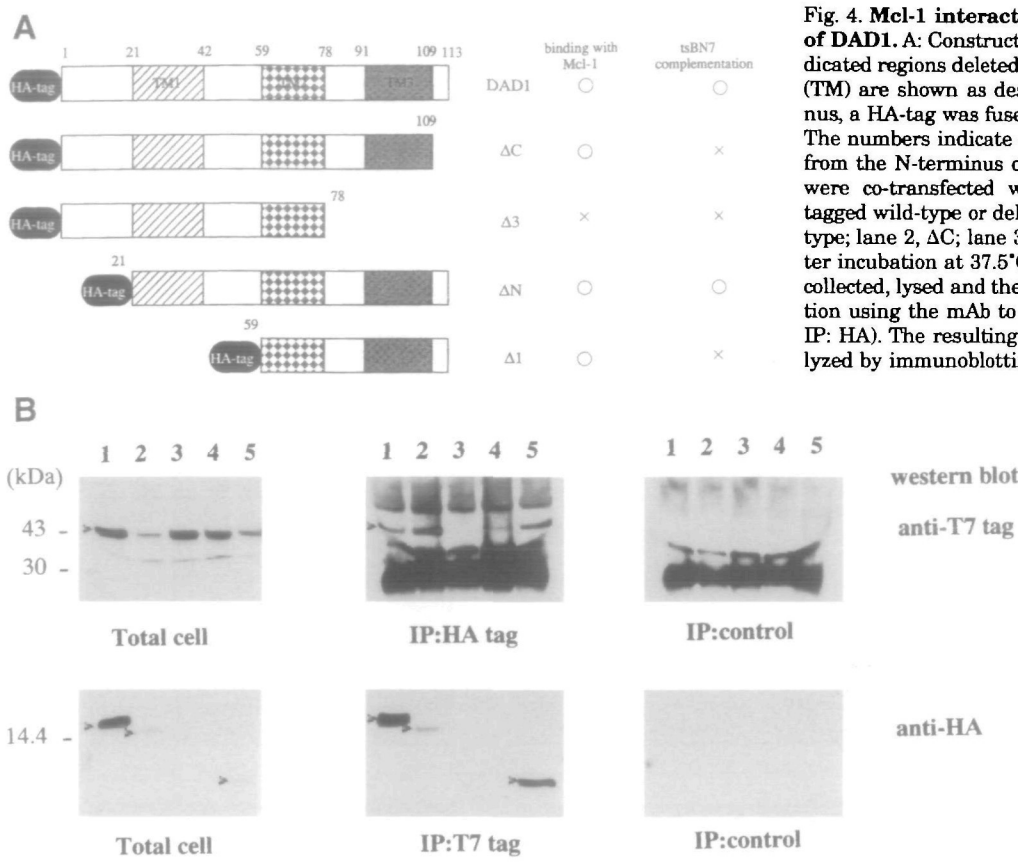

Fig. 4. **Mcl-1 interacts with the C-terminal region of DAD1.** A: Construction of DAD1 cDNAs with the indicated regions deleted. Three transmembrane domains (TM) are shown as described (*15*, *25*). At the N-terminus, a HA-tag was fused in frame to the DAD1 cDNAs. The numbers indicate the amino acid residue numbers from the N-terminus of DAD1. B: 2×10^6 COS-7 cells were co-transfected with T7-tagged Mcl-1 and HA-tagged wild-type or deleted DAD1 cDNAs (lane 1, wild-type; lane 2, ΔC; lane 3, Δ3, lane 4, ΔN; lane 5, Δ1). After incubation at 37.5°C for 48 h, transfected cells were collected, lysed and then subjected to immunoprecipitation using the mAb to the T7-tag or HA-tag (IP: T7 or IP: HA). The resulting precipitated samples were analyzed by immunoblotting using the mAb to the HA-tag (anti-HA) or T7-tag (anti-T7 tag). As controls, total lysates were analysed by immunoblotting with the mAb to the T7-tag or HA-tag as indicated.

with either the mAb to T7 or the antibodies to Mcl-1 (Fig. 5). DAD1 showed a reticular pattern, characteristic of the endoplasmic reticulum. On the other hand, Mcl-1 showed a reticular pattern with some speckles throughout the cytoplasm as described (*6*, *19*, *20*). When superimposed, the DAD1 and Mcl-1 proteins partly overlapped.

DISCUSSION

One of the bcl-2 family member proteins, Mcl-1, was identified as a DAD1-binding protein. Since the full length Mcl-1 and ΔN-Mcl-1 both interact with DAD1 *in vivo*, the inability of the full length Mcl-1 to bind to DAD1 observed on two-hybrid analysis may reflect structural hindrance of the N-terminal region of Mcl-1, which is not essential for binding to DAD1. Although Mcl-1 has been reported to be mainly present in the mitochondria (*19*, *20*), some exists in the light membrane fraction (*6*) that contains the ER, in which DAD1 is localized (*15*). DAD1 binds to the C-terminal region of Mcl-1 containing BH^2 out of the four BH domains. Taking this together the report that the BH region of bcl-2 is a receptor domain which plays an important role in binding with other proteins (*22*), DAD1 may bind to Mcl-1 through BH^2, anchoring Mcl-1 in the ER where DAD1 is localized (*15*).

We first thought that DAD1 may have a dual function, that is, inhibition of apoptosis through Mcl-1 and *N*-linked glycosylation. In this regard, we expected that overexpression of ΔC-DAD1 and Δ1-DAD1, both of which bind to Mcl-1, may prevent apoptosis caused by the tsBN7 mutation (*23*), although neither cDNA can confer the ts^+ phenotype to tsBN7 cells. They did not prevent apoptosis (data not shown). In contrast to our initial idea, thus, Mcl-1 may be involved in a cellular defense pathway which prevents apoptosis caused by loss of *N*-linked glycosylation.

It is noticeable that ΔC-DAD1 lacking only four amino acid residues from the C-terminus did not complement the tsBN7 mutation, while ΔN-DAD1 lacking 20 amino acid residues from the N-terminus rescued the ts growth of tsBN7 cells. The inability of ΔC-DAD1 to complement the tsBN7 mutation may not be due to poor expression of ΔC-DAD1 proteins, because ΔC-DAD1 proteins were apparently expressed in COS cells, although ΔN-DAD1 was not detected under the same conditions. In COS cells, DAD1 can be transiently expressed. However, DAD1 seems to be unstable or toxic in cells, since transformants of the BHK21 cell line, which stably express extragenic DAD1 proteins, appeared very rarely. Thus, we could not establish tsBN7 cell lines stably expressing ΔC-DAD1, although these transformants are important for distinguishing apoptosis from the *N*-linked gylcosylation defect.

There has been a report that the N-terminal 28 amino acids of the DAD1 protein bind with the short cytoplasmic tail of another subunit of the mammalian oligosaccharyltransferase, OST 48 (*24*). Taking this previous report together with the fact that ΔN-DAD1 lacking the N-terminal 21 amino acid residues complements the tsBN7 mutation, the OST48 protein may not be essential for the oligosaccharyltransferase activity. While the N-terminus of the DAD1 protein is predicted to be located in the cytoplasm (*15*, *25*),

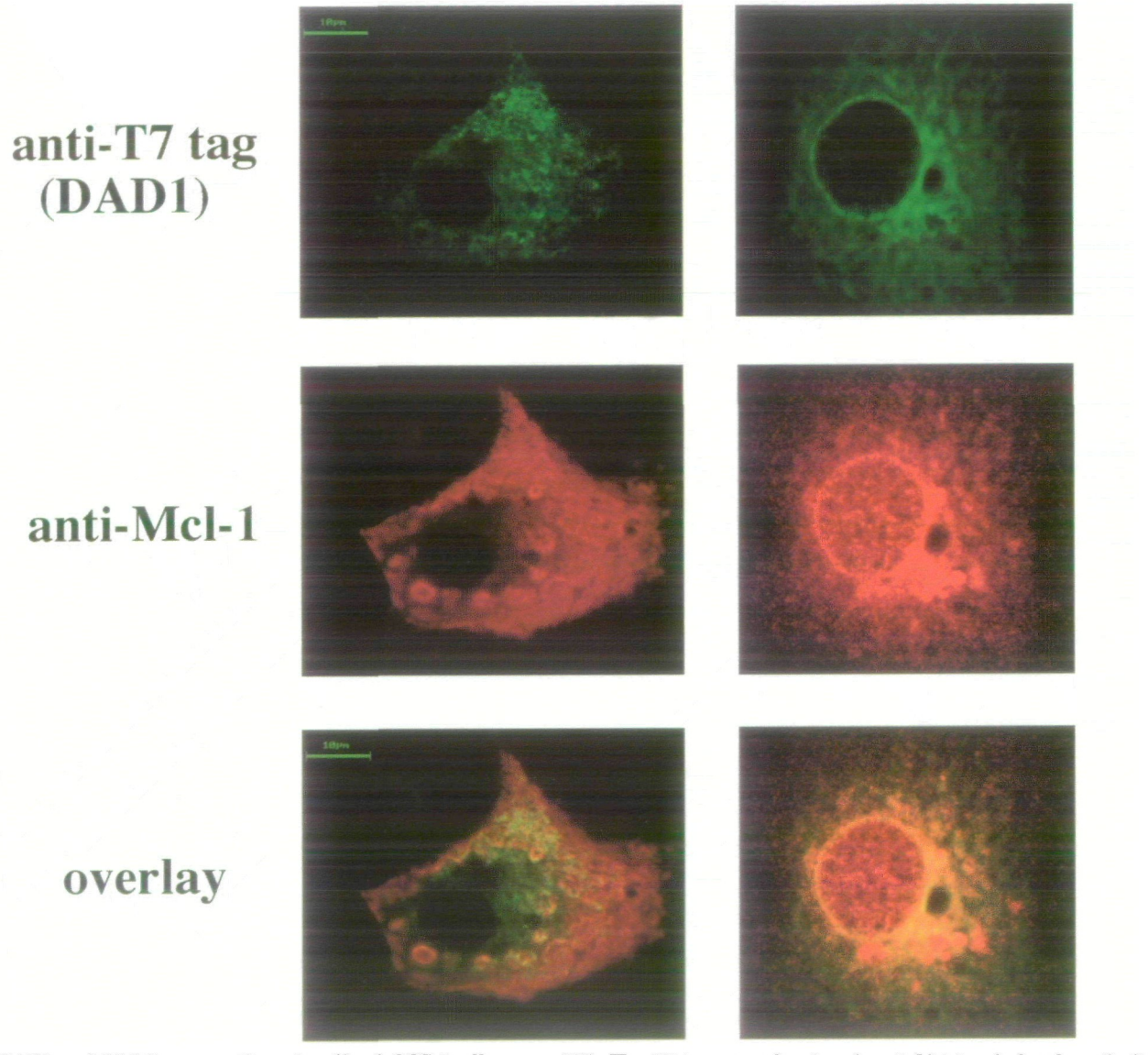

Fig. 5. **DAD1 and Mcl-1 are partly colocalized.** COS-7 cells were co-transfected with the T7-tagged DAD1 and wild-type Mcl-1 cDNAs. After incubation at 37.5°C for 48 h, transfected cells were fixed with methanol and then stained with the indicated antibodies as described (*15*). The T7-tag monoclonal and anti–Mcl-1 polyclonal antibodies were used as primary antibodies. Two different cells are shown. Bar = 10 μm.

TABLE II. **Ability of deleted DAD1 cDNA to complement the tsBN7 mutation.**

DAD1 clones	Number of colonies/2 × 10^4 cells	
	33.5°C + hygromycin B	39.5°C
HA-DAD1	119	142
HA-ΔDAD1	32	0
HA-Δ3DAD1	92	0
HA-ΔNDAD1	94	143
HA-Δ1DAD1	113	0
Vector	112	0

its real function remains to be investigated.

We wish to thank Dr. S.J. Elledge for the cDNA library of human B cells.

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