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13. ABSTRACT ***(Maximum 200 Words)***

The BRCA1 C-Terminal (BRCT) domain is present in a number of proteins that are involved in various aspects of chromosomal events. The BRCT domain of BRCA1 is important for BRCA1 function in DNA repair and transcriptional activation. When tethered to chromosomal DNA, this region of BRCA1 is capable of inducing changes in chromatin structure. Despite the sequence homology and functional proximity shared by the BRCT-containing proteins, it is not known whether different BRCT domains confer a common biochemical activity. Here we show that the BRCT domain of the *Saccharomyces cerevisiae* RAP1 (scRAP1), but not the other yeast BRCT domains tested, is capable of activating transcription and remodeling chromatin in a manner similar to that shown for the BRCT domain of BRCA1. A number of the highly conserved amino acid residues in the BRCT domains of RAP1 and BRCA1 are critical for their activities in yeast and human cells, respectively. Our work also reveals a region in the scRAP1 BRCT domain that negatively regulates its transcriptional activity. We suggest that chromatin remodeling is an important feature shared by a subset of the BRCT family members to facilitate multiple chromosomal events.

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INTRODUCTION

Mutations of BRCA1 account for a large proportion of familial breast and ovarian cancers (1) (2). While the exact biochemical function of the BRCA1 protein remains to be elucidated, multiple lines of evidence suggest that it is involved in regulation of several nuclear functions including transcription, DNA repair, recombination, and checkpoint control (3) (4) (5) (6) (7) (8) (9) (10) (11). The entire 1863 amino acid protein contains a highly conserved RING finger domain at the amino terminus and two repeats of the BRCT (BRCA1 C-terminus) domain at the carboxyl terminus. While most cancer-predisposing mutations of BRCA1 result in gross truncation of the protein, 5-10% of the disease-associated mutations lead to single amino acid substitutions, many of which occur in the RING or BRCT domains(12). This suggests that both domains are pivotal to BRCA1 function in tumor suppression.

The BRCT domain of BRCA1 is required for BRCA1 function in both transcriptional activation and DNA repair (3) (4) (5) (6) (7) (8) (9) (10). When tethered via the GAL4 DNA binding domain (DBD), this domain is capable of stimulating transcription from a GAL4-responsive reporter gene (3) (4) and remodeling chromatin from chromosomally embedded GAL4 binding sites (13). The same region is also reported to interact with histone modifying enzymes such as the histone acetyltransferase p300 (14) (15) and the human histone deacetylase HDAC (16). Importantly, cancer-predisposing mutations in this region abolish the ability of BRCA1 to activate transcription, enhance DNA repair efficiency, and remodel chromatin. Thus, BRCA1 may utilize the BRCT domain to increase chromatin accessibility and facilitate multiple chromosomal events.

The BRCT domain, first identified in BRCA1, is an evolutionarily conserved motif found in a large number of proteins that are involved in various aspects of chromosomal events, such as DNA repair, replication, recombination, gene activation and silencing, and checkpoint control (17) (18). The functional proximity of the BRCT superfamily raises the possibility that BRCT domains may share a common biochemical property, such as transcriptional and chromatin remodeling as demonstrated for the BRCA1 BRCT domain. Several recent reports suggest a role of the BRCT domains in mediating protein-protein interactions with another BRCT domain or a different structural module. For example, the BRCT domains present in DNA ligase III and XRCC1, two mammalian DNA repair proteins, interact with each other strongly (19). In addition, the BRCT domain of BRCA1 binds CtIP, a transcriptional co-repressor (20) (21) (22). Despite these findings, it remains to be determined whether BRCT domains utilize a common biochemical feature to regulate a specific nuclear process.

The overall objectives of the study are to determine the critical amino acid residues that are critical for BRCA1 function in chromatin remodeling and to identify the target proteins that mediate the function of chromatin remodeling. During the first year of the study, we compared the BRCT domains of human BRCA1 and a number of yeast proteins for their potential to stimulate transcription and induce changes in chromatin structure. Our work shows that the BRCT domain of the *Saccharomyces cerevisiae* RAP1 (scRAP1), but not the other yeast BRCT domains tested, displays in vivo properties similar to those shown for the BRCT domain of BRCA1. Furthermore, a comprehensive mutational study demonstrates that a number of the highly conserved residues in the BRCT domains of scRAP1 and human BRCA1 are important for transcription activation by these BRCT domains.

BODY OF THE REPORT

The scRAP1 BRCT Domain is a Transcription Activation Domain---To test the possibility that BRCT domains besides that of BRCA1 may also be capable of activating transcription and remodeling chromatin, we fused the hemagglutinin (HA)-tagged GAL4-DBD to the BRCT domains of several yeast proteins (Figure 1a). The BRCT-containing proteins chosen here are

known for their roles in regulation of various aspects of chromosomal events. These include Dpb11p involved in DNA replication and S-phase checkpoint (23); Rfc1p in DNA replication and repair (24); Rad9p in DNA damage checkpoint (25); Rap1p in transcriptional activation, silencing, and telomere length maintenance (26) (27); and Esc4p in gene silencing (17). As shown in Figure 1b, all GAL4 fusion proteins were expressed with the expected sizes and at a comparable level in yeast.

The potentials of these fusion proteins to activate transcription were analyzed using a GAL4-responsive lacZ reporter gene (Figure 1c). Consistent with previous findings, GAL4-BRCA1 activated transcription in yeast as well as human cells (compare column 1 and 2 in Figure 1c; also see Figure 4). Of all the other BRCT fusion proteins tested, only GAL4-RAP1 gave rise to an elevated β-galactosidase activity (compare column 1 with 7), albeit to a lesser extent than GAL4-BRCA1. None of the remaining GAL4-BRCT constructs showed any appreciable levels of transcriptional stimulation (columns 3-6, and 8). While it remains possible that the lack of transcription activation may be due to improper folding of the BRCT domains in the GAL4 fusion context, our data suggest that at least a subset of the BRCT domains can act as transcriptional activation domains.

The scRAP1 BRCT Domain can Remodel Chromatin---To explore the mechanisms by which the scRAP1 BRCT domain activated transcription, we examined the effect of the GAL4 derivatives on chromatin structure. The yeast strain used in this study contains five GAL4 binding sites engineered near the autonomously replicating sequence 1(ARS1) on chromosome IV (28). It has been shown that, while all GAL4 derivatives can get access to the chromosomally embedded GAL4 binding sites, only those that activate transcription and chromosomal replication are capable of inducing distinct changes in the micrococcal nuclease (MNase) digestion pattern around the GAL4 binding sites (13). As shown in Figure 2, tethering of the BRCT domain of BRCA1 to the chromosome changed the nuclease digestion pattern around the GAL4 binding sites, namely, the intensity of band A was attenuated whereas that of band B enhanced (indicated by arrows; compare lanes 1-2 with 3-4). The same change was observed with GAL4-RAP1 (lanes 13 and 14), but not with any of the transcriptionally inactive GAL4-BRCT fusion proteins (lanes 5-12, 15-16). Thus, transcriptional activation by the BRCT domains correlates with their ability to induce changes in chromatin structure.

The observed changes in chromatin structure are unlikely due to the presence of the entire transcription machinery or initiation of transcription *per se* in the vicinity of the GAL4 sites, as the GAL4 binding sites are located at least 800 bp away from the nearest transcriptional promoter. Rather, a chromatin remodeling complex may be recruited by the scRAP1 BRCT domain to induce the changes in the local chromatin structure. In keeping with this notion, the BRCT domain of BRCA1 has been shown to associate with certain histone modifying enzymes such as the histone acetyltransferase p300 (14) (15) and the human histone deacetylase HDAC (16). Taken together, the transcription activation and chromatin remodeling assays may provide useful tools for identifying additional BRCT domains that share the same biochemical features as those of scRAP1 and BRCA1.

The Conserved Residues in the BRCA1 BRCT Domain are Important for Transcriptional Activation--- To address the significance of the conserved residues in transcriptional activation, we introduced mutations in several conserved blocks of the second BRCT domain of BRCA1 (HsBRCA1-b in Figure 3). These include the N-terminal tail, α1, α3, and the C-terminal tail as predicted based on the structure of the XRCC1 BRCT domain (29). The four-stranded β sheet and α2 were not targeted for mutagenesis in this study, as the β sheet is predicted to form the core of the structure and the corresponding mutant proteins tend to be unstable (data not shown), and the sequence for α2 is not universally present in the BRCT superfamily.

A total of eleven alanine substitution mutants were constructed in the context of the GAL4-BRCA1 fusion. To analyze the mutational effects in a more physiological context, the wild type and mutant fusion proteins were expressed and their transcriptional activity was assessed in human 293T cells (Figure 4a and b). As expected, the wild-type GAL4-BRCA1 fusion can potently stimulate transcription from a GAL4-responsive luciferase reporter construct (compare lane 2 with 1 in Figure 4b). Mutations at multiple conserved amino acid residues either reduced or completely abolished the transcriptional activity. For example, IF1760-1AA of the N-terminal tail, L1780A and MV1783-4AA of α1, G1788A between α1 and β2, VL1838-9AA and S1841A of α3, and YLI1853-5AAA of the C-terminal tail all severely impaired the *trans*-activation capability of the BRCT domain (compare lane 2 with lanes 3, 6, 8, 9, 10, 11 and 13 in Figure 4b). Of all the conserved residues mutated in this study, only L1764A failed to affect BRCT function in a significant manner (lane 4 in Figure 4b). In addition, mutations of three non-conserved residues (D1778A, W1782A, and Q1848A) did not cause deleterious effects either (lanes 5, 7, and 12), despite the fact that the corresponding residues of D1778 and W1782 in XRCC1 are involved in dimer formation between two BRCT domains (29). The results are also summarized below the sequence of the BRCT domain in Figure 3.

Natural mutations at M1783, G1788, and S1841 have been identified as unclassified mutant variants in terms of cancer predisposition (12) and predicted to affect protein folding (29). The alanine substitution mutants at these three positions indeed abrogated the transcriptional activation by GAL4-BRCA1 (lanes 8, 9, and 11 in Figure 4b), suggesting that the corresponding natural mutations at these sites may represent genuine cancer-predisposing mutations. Taken together, the data strongly suggest that a majority of the conserved amino acid residues in the BRCT domain are critical for BRCA1 function in transcriptional activation. Our results in human cells are also in agreement with a recent yeast-based study of the BRCA1 BRCT domain (30). In particular, the three regions mutagenized in both studies (F1761, L1780, and YLI1853-5) are important for *trans*-activation in human as well as yeast cells.

Evidence for a Inhibitory Region in the scRAP1 BRCT Domain---Next, we asked whether the conserved residues in the scRAP1 BRCT domain are important for transcriptional activation as well. In addition to the BRCT domain in the N-terminus of scRAP1, the protein also contains several other domains, such as the central Myb-like DNA binding domain and the *trans*-activation and silencing domains in the C-terminus (31). A human homologue of scRAP1 (hRAP1) was recently identified (32). While hRAP1 is highly diverged from its counterparts in *Scaccharomyces cerevisiae* (scRAP1) and *Kluyveromyces lactis* (klRAP1), the BRCT domain, the Myb-related motif, and the silencing domain are conserved (see alignment of the BRCT domain in Figure 3). Interestingly, the potent *trans*-activation domain identified in scRAP1 and klRAP1 is not present as such in hRAP1 (32). To ascertain the relevance of the BRCT motif to the transcriptional activation conferred by GAL4-RAP1, we changed several amino acid residues in the regions that correspond to those in the BRCA1 BRCT domain studied above. As illustrated in Figure 3, alanine substitutions were introduced at PL125-6 of the N-terminus; L-143, N-144, L149, R151, and LI152-3 of α1; GG157-8 between α1 and β2; Y190, IK191-2, and C194 of α3; and YLV205-8 of the C-terminal tail.

As shown in Figure 5, two mutations in α3 (Y190A and C194A) and one in the C-terminal tail (YLV205-8AAA) either reduced or completely abolished the ability of GAL4-RAP1 to stimulate transcription (lanes 5, 6 and 7). Notably, the corresponding residues in the BRCA1 BRCT domain had a similar deleterious effect on transcriptional activation (see W1837R in ref. (13); and S1841A and YLI1853-5AAA in Figure 4). Therefore, the conserved residues toward the end of both BRCT domains are important for the transcriptional activation function. However, mutations at several other conserved positions of the scRAP1 BRCT domain yielded a quite unexpected result. Instead of impairing the function of the BRCT domain, these mutations actually significantly increased the transcriptional activity of GAL4-RAP1 (compare lane 8 with 9-13 of

Figure 5; also note the two scales for lanes 1-7 and lanes 8-13). Most strikingly, PL125-6AA of the N-terminus, LI152-3AA of α1, and GG157-8AA of the loop between α1 and β2 were at least 15 fold as robust as the wild-type protein (compare lane 8 with 9, 11, and 12 of Figure 5). Thus, these conserved residues may constitute a region that negatively modulates the BRCT function. This feature seems to be unique to the BRCT domain of scRAP1, as mutations at the corresponding residues in the BRCT domain of BRCA1 had the opposite effect on transcriptional activation in human cells (Figure 4). The mutational results are also summarized with signs above the sequence of the scRAP1 BRCT domain in Figure 3.

Based on the crystal structure of the XRCC1 BRCT domain (29), the residues involved in repressing the BRCT function in scRAP1 (PL125-6, L149, LI152-3, GG157-8, and IK191-2) are predicted to reside proximally in the tertiary structure. Moreover, L149 and LI152-3 of α1 and IK191-2 of α3 are likely to be involved in mediating the intra-molecular interaction between α1 and α3, whereas the highly conserved Gly-Gly residues between α1 and β2 may be important for proper orientation of the two helices. Thus, it is conceivable that the interaction between α1 and α3 may result in a conformation that is unfavorable for transcriptional activation. Alternatively, the amino acid residues of interest may be involved in an interaction with a transcriptional repressor. In either situation, the negative regulatory region of the scRAP1 BRCT domain may serve as a molecular switch that coordinates the multiple functions of RAP1 in transcriptional activation, gene silencing (26), and telomere length maintenance (33), etc.

The BRCT domain is identified by sequence comparison, yet its role in the biological function of the full-length protein remains obscure. In fact, the entire N-terminal sequence including the BRCT domain is not essential for scRAP1 to support cell viability (34). In this regard, it is somewhat puzzling that the BRCT domain is well conserved between yeast and human, whereas the sequence for the potent *trans*-activation domain found in scRAP1 and klRAP1 is not (32). While the exact contribution of the BRCT domain to RAP1 function awaits further investigation, it is possible that the transcriptional activation and chromatin remodeling property associated with the BRCT domain may be important for a more specialized, non-essential function of scRAP1. It is also tempting to speculate that, in the absence of a strong *trans*-activation domain in hRAP1, the BRCT domain may play a more prominent role in RAP1-mediated regulation of chromosomal events in human cells.

KEY RESEARCH ACCOMPLISHMENTS

1) Identification of a subset of BRCT domains that are capable of remodeling chromatin, activating transcription and DNA replication.

2) Determination of key residues in the BRCT domains that are essential for the functions in chromatin remodeling and activation of transcription.

3) Establishment of genetic screens for target proteins that mediate the functions of the BRCT domain.

4) Initiation of the investigation of BRCA1 function in regulation of multiple nuclear events including DNA replication.

REPORTABLE OUTCOMES

Tsuyoshi Miyake, Yan-Fen Hu, David S. Yu, Rong Li. (2000) Transcriptional activation and chromatin remodeling by the BRCA1 C-terminus (BRCT) domains. Manuscript submitted.

CONCLUSIONS

The BRCA1 C-terminal (BRCT) motif is present in a number of proteins that are implicated in regulation of various chromosomal events. However, it is not clear whether the BRCT family members share a common biochemical property. Our work provides strong in vivo evidence that at least a subset of the BRCT domains, i.e., those of BRCA1 and scRAP1, can act as transcription activation and chromatin remodeling domains. This represents an important progress in understanding the functional similarity of the BRCT domains. Furthermore, our work shows that the conserved amino acid residues in the BRCT domains are critical for their functions in chromatin remodeling and transcription activation.

The yeast RAP1 is involved in multiple nuclear processes including transcriptional activation, gene silencing, replication, and telomere length maintenance. Furthermore, the recent identification of the human RAP1 shows a high degree of conservation of the BRCT domain between human and yeast. Our study also raises an intriguing possibility that the BRCT domain may serve as a molecular switch to coordinate multiple RAP1-mediated chromosomal events.

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APPENDICES

Figure 1. Activation of transcription by various GAL4-BRCT fusion proteins. **a.** Diagram illustrating the relative position of the BRCT domains in BRCA1 and the yeast proteins. Each shaded region represents one BRCT motif. The regions marked by brackets are included in the same GAL4 fusion protein. **b.** The HA-tagged GAL4 derivatives were detected by immunoblotting of an equal amount of whole cell extracts. The molecular weight markers are indicated on the left. Asterisks indicate the bands representing the full-length fusion proteins. See the text for more description of each fusion protein. **c.** The β-galactosidase activity from cells that express various GAL4-BRCT fusion proteins. The results shown are an average of data from at least four independent experiments.

Figure 2. The BRCT domains from BRCA1 and scRAP1 can induce changes of the chromatin structure in vivo. Indirect end-labeling assay was used for the analysis of the MNase digestion pattern around the chromosomally embedded GAL4 binding sites. Two time points of the MNase treatment are shown for each GAL4 derivative. The EcoR1 fragment containing the *ARS1* locus was revealed by a radioactive probe derived from an NheI-EcoR1 restriction fragment (indicated by a thick bar on the left). The arrows (A and B) indicate the two bands the intensities of which were most significantly affected by GAL4-BRCA1 and GAL-RAP1. The approximate positions of the GAL4 binding sites and the four *cis*-elements of *ARS1* are indicated on the left.

Figure 3. Diagram showing the primary sequence and the predicted secondary structure of the BRCT domains. The highly conserved amino acid residues are highlighted in bold and shade, and the similar residues in bold only. The numbers on the top and bottom of the sequences correspond to the positions of amino acids in scRAP1 and hBRCA1, respectively. Asterisks and triangles indicate those amino acids that are mutated in this study. Asterisks are those that show no obvious phenotypes. Triangles pointing up designate mutants that cause "super-activation", and those pointing down indicate mutations that reduce transcriptional activity.

Figure 4. Mutational analysis of the second BRCT domain of BRCA1 in a human cell-based transcription assay. **a.** Expression of the HA-tagged GAL4 –BRCA1 chimeras in human 293T cells. The immunoblot is probed with an anti-HA antibody (12CA5). **b.** Luciferase assays are performed using cell lysates from human 293T cells transfected with a luciferase reporter, a β-

galactosidase (as an internal control) reporter, and the GAL4-BRCA1 expression vectors. The transcriptional activity is expressed as fold of activation.

Figure 5. Mutational analysis of the scRAP1 BRCT domain using a yeast-based transcription assay. Liquid β-galactosidase assays are carried out using lysates from yeast cells that express various GAL4 derivatives. For a convenient comparison, β-galactosidase value for the GAL4-derivatives are shown in two different scales (left and right panels). The wild-type activity is set as 100%.

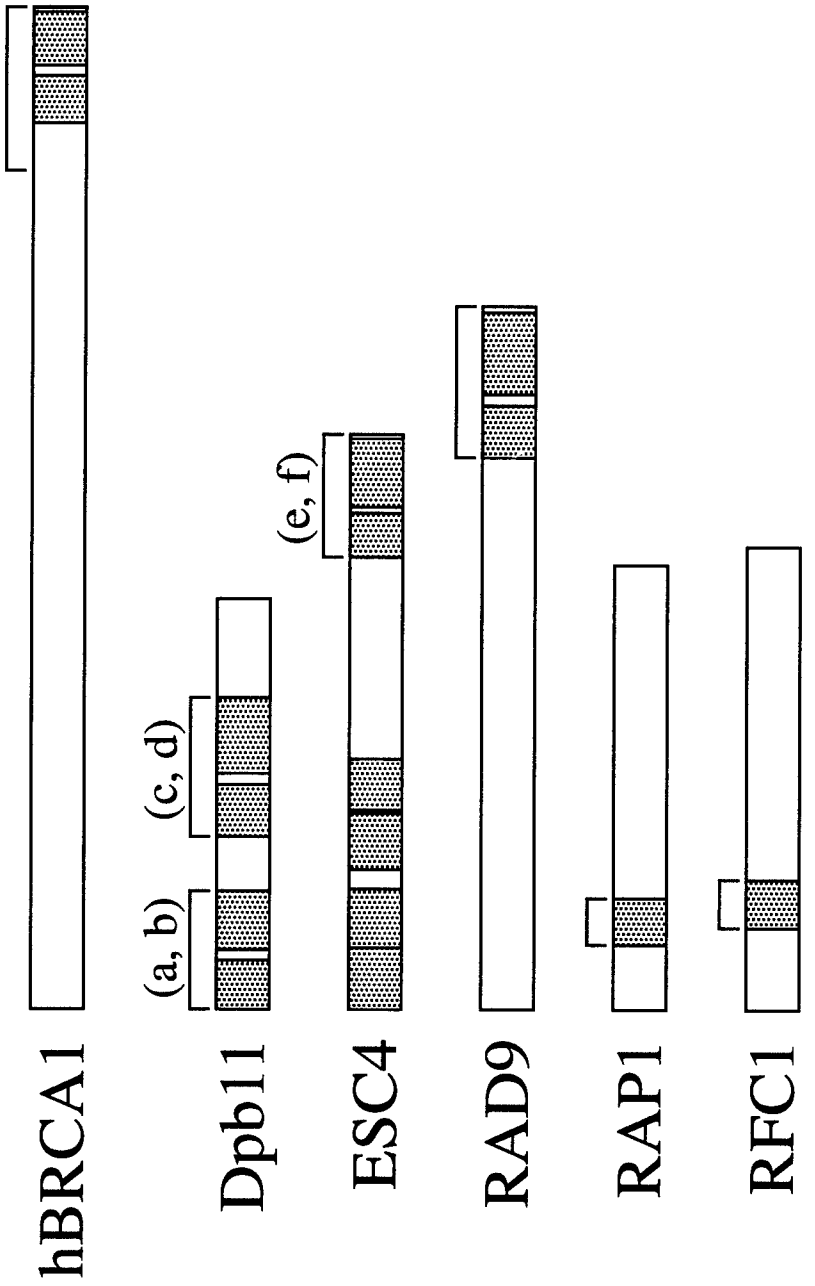
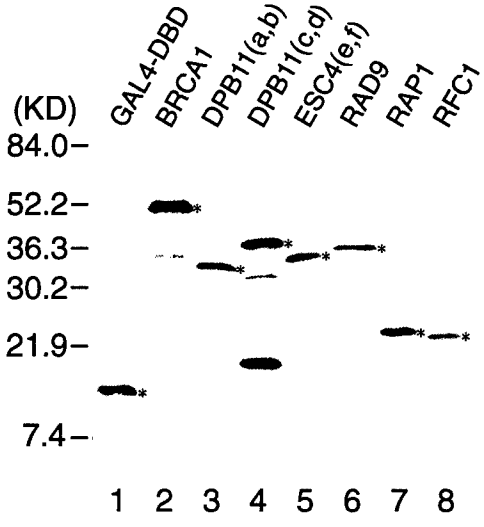


Fig. 1A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RAD9
RAP1
RFC1
1 2 3 4 5 6 7 8

Fig.1B

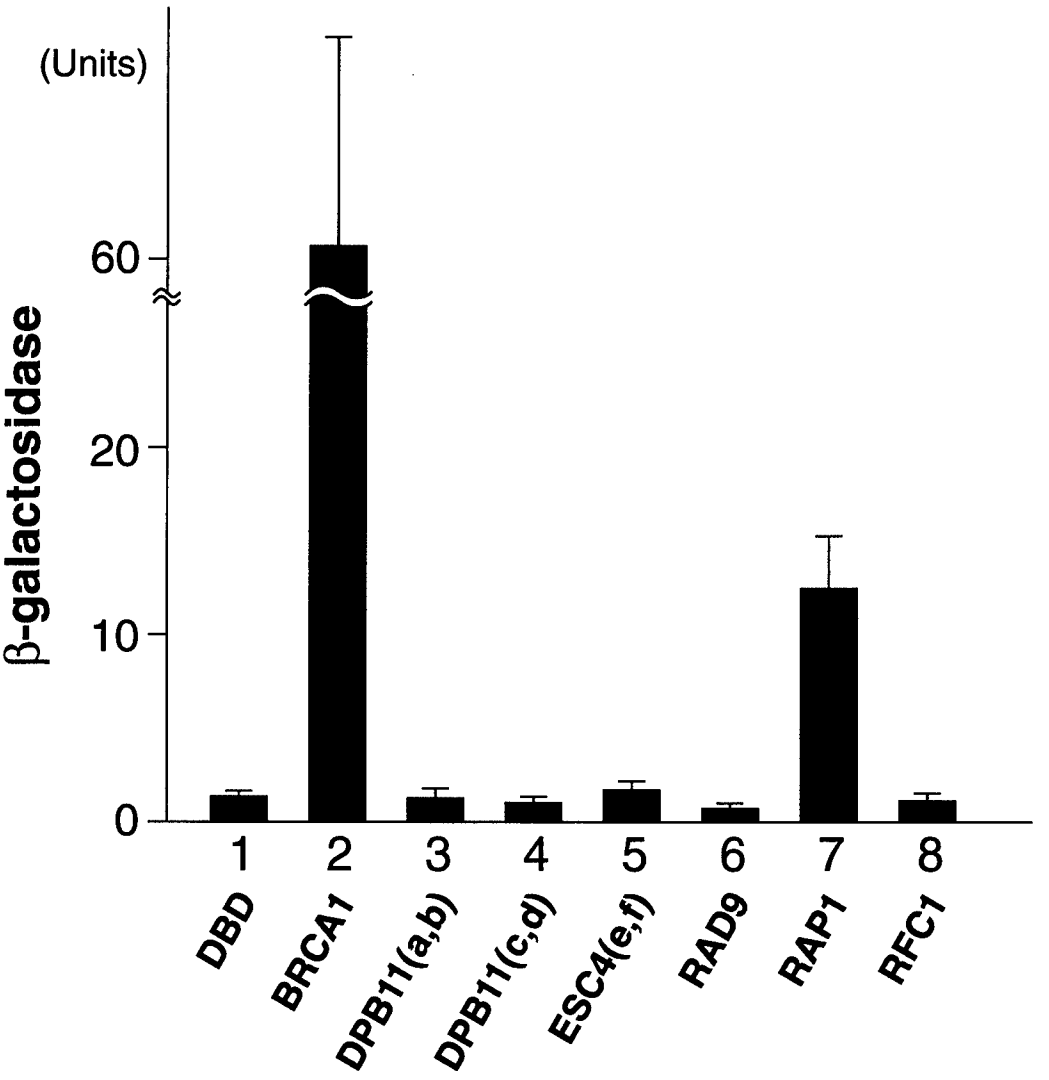

(Units)
β-galactosidase
60
20
10
0
1
2
3
4
5
6
7
8
DBD
BRCA1
DPB11(a,b)
DPB11(c,d)
ESC4(e,f)
RAD9
RAP1
RFC1

Fig.1C

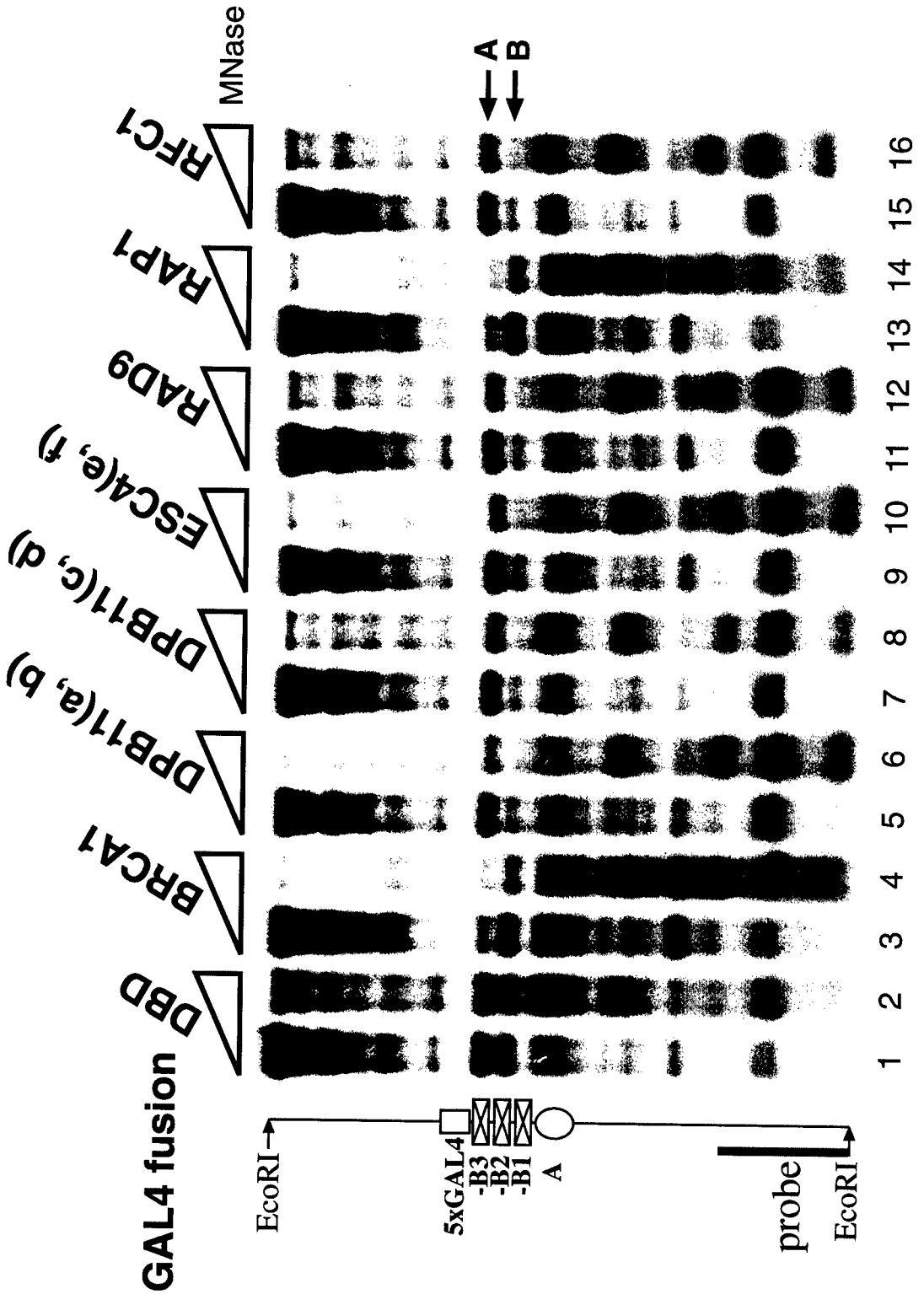

GAL4 fusion
DBD
BRCA1
DPB11(a, b)
DPB11(c, d)
ESC4(e, f)
RAD9
RAP1
RFC1
MNase
A
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
EcoRI
5xGAL4
-B3
-B2
-B1
A
probe
EcoRI

Fig.2

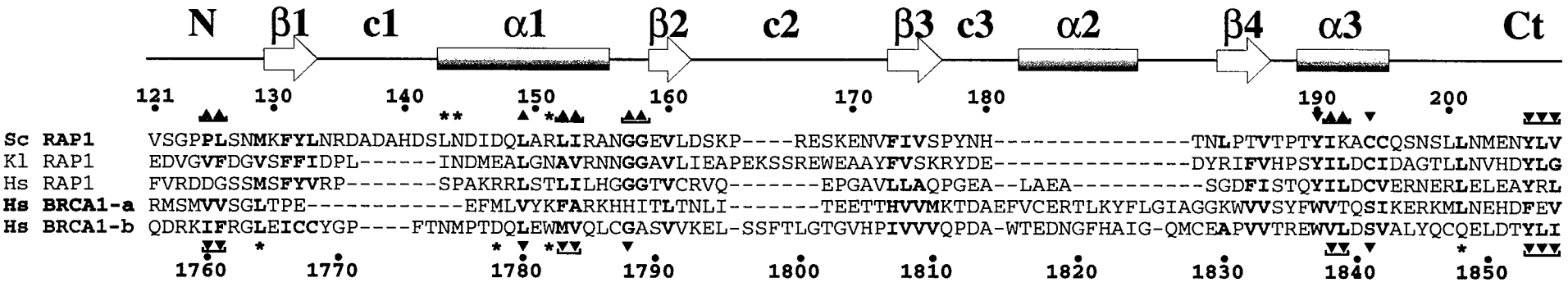
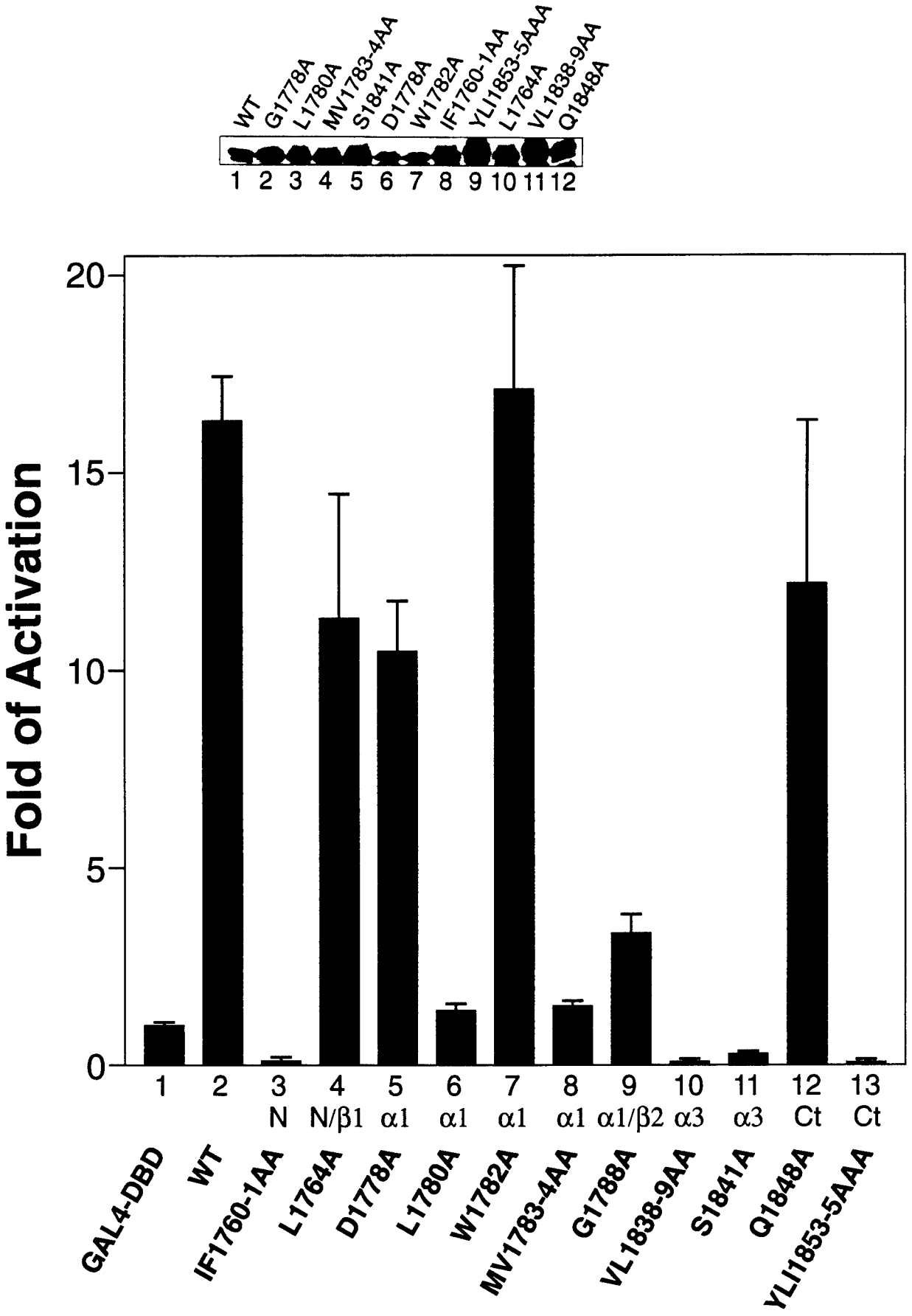

N β1 c1 α1 β2 c2 β3 c3 α2 β4 α3 Ct
121 130 140 150 160 170 180 190 200
Sc RAP1 VSGPPLSNMKFYLNRDADAHDSLNDIDQLARLIRANGGEVLDSKP----RESKENVFIVSPYNH----------TNLPTVTPTYIKACCQSNSLLNMENYLV
Kl RAP1 EDVGVFDGVSFFIDPL------INDMEALGNAVRNNGGAVLIEAPEKSSREWEAAYFVSKRYDE----------DYRIFVHPSYILDCIDAGTLLNVHDYLG
Hs RAP1 FVRDDGSSMSFYVRP-------SPAKRRLSTLILHGGGTVCRVQ-------EPGAVLLAQPGEA--LAEA----------SGDFISTQYILDCVERNERLELEAYRL
Hs BRCA1-a RMSMVVSGLTPE----------EFMLVYKFARKHHITLTNLI-------TEETTHVVMKTDAEFVCERTLKYFLGIAGGKWVVSYFWVTQSIKERKMLNEHDFEV
Hs BRCA1-b QDRKIFRGLEICCYGP----FTNMPTDQLEWMVQLCGASVVKEL-SSFTLGTGVHPIVVVQPDA-WTEDNGFHAIG-QMCEAPVVTREWVLDSVALYQCQELDTYLI
1760 1770 1780 1790 1800 1810 1820 1830 1840 1850

Fig. 3


WT
G1778A
L1780A
MV1783-4AA
S1841A
D1778A
W1782A
IF1760-1AA
YLI1853-5AAA
L1764A
VL1838-9AA
Q1848A
1 2 3 4 5 6 7 8 9 10 11 12
Fold of Activation
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13
N N/β1 α1 α1 α1 α1 α1/β2 α3 α3 Ct Ct
GAL4-DBD
WT
IF1760-1AA
L1764A
D1778A
L1780A
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VL1838-9AA
S1841A
Q1848A
YLI1853-5AAA

Fig. 4A,B

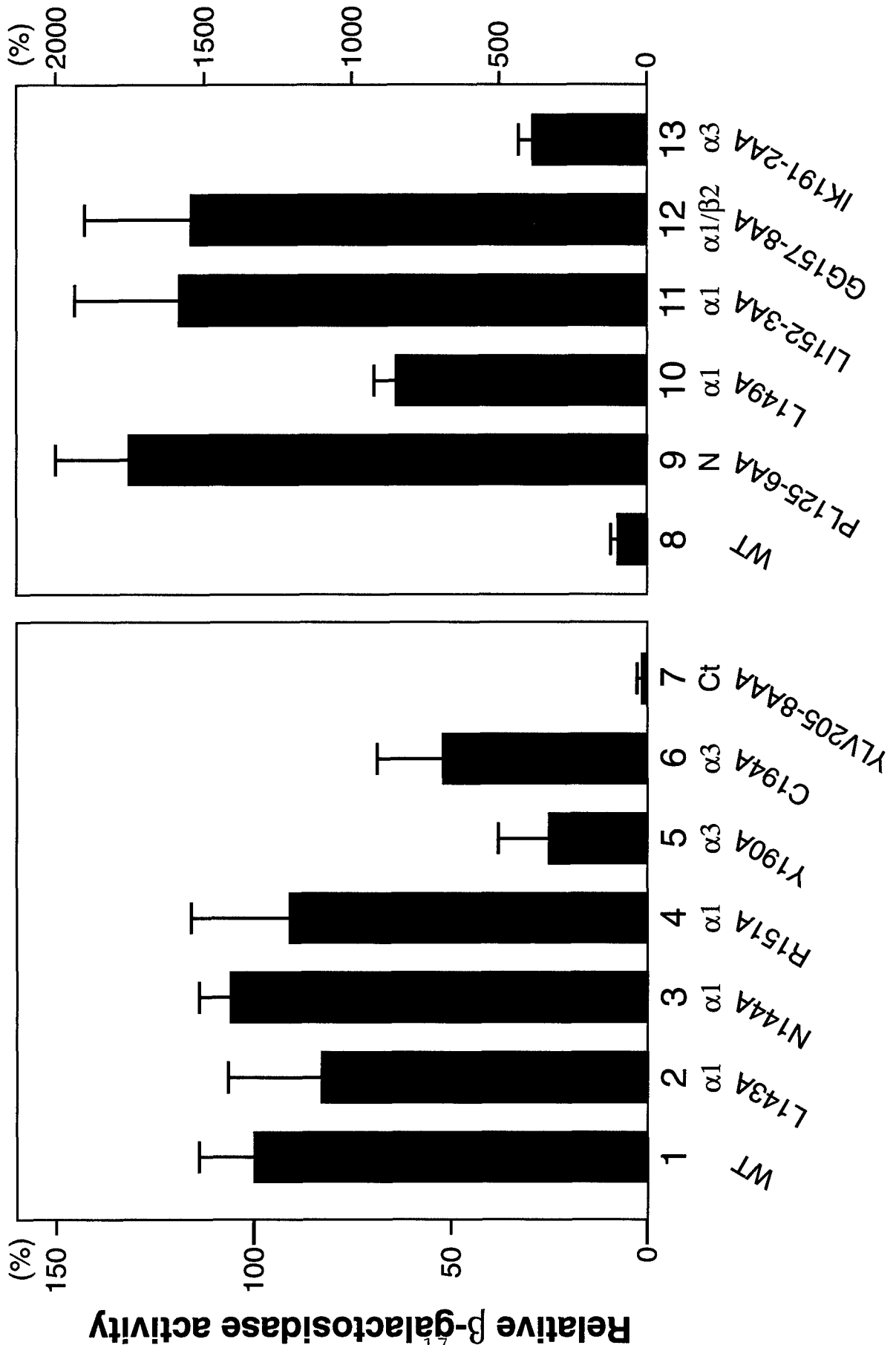
Relative β-galactosidase activity
(%)
150
100
50
0
(%)
2000
1500
1000
500
0
1 WT
2 L143A α1
3 N144A α1
4 R151A α1
5 Y190A α3
6 C194A α3
7 Ct YLV205-8AAA
8 WT
9 N PL125-6AA
10 L149A α1
11 LI152-3AA α1
12 GG157-8AA α1/β2
13 α3 IK191-2AA

Fig.5

Transcriptional Activation and Chromatin Remodeling by the BRCA1 C-Terminal (BRCT) Domains

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Running Title: BRCT Domains of yeast RAP1 and human BRCA1

The BRCA1 C-terminal (BRCT) domain is present in a number of proteins that are involved in various aspects of chromosomal events (1, 2). The BRCT domain of BRCA1 is important for BRCA1 function in DNA repair (3-6) and transcriptional activation (7-10). When tethered to chromosomal DNA, this region of BRCA1 is capable of inducing changes in chromatin structure (11). Despite the sequence homology and functional proximity shared by the BRCT-containing proteins, it is not known whether different BRCT domains confer a common biochemical activity. Here we show that the BRCT domain of the *Saccharomyces cerevisiae* RAP1 (scRAP1), but not the other yeast BRCT domains tested, is capable of activating transcription and remodeling chromatin in a manner similar to that shown for the BRCT domain of BRCA1. A number of the highly conserved amino acid residues in the BRCT domains of RAP1 and BRCA1 are critical for their activities in yeast and human cells, respectively. Our work also reveals a region in the scRAP1 BRCT domain that negatively regulates its transcriptional activity. We suggest that chromatin remodeling is an important feature shared by a subset of the BRCT family members to facilitate multiple chromosomal events.

INTRODUCTION

Mutations of BRCA1 account for a large proportion of familial breast and ovarian cancers (12, 13). While the exact biochemical function of the BRCA1 protein remains to be elucidated, multiple lines of evidence suggest that it is involved in regulation of several nuclear functions including transcription, DNA repair, recombination, and checkpoint control (3-10, 14). The entire 1863 amino acid protein contains a highly conserved RING finger domain at the amino terminus and two repeats of the BRCT (BRCA1 C-terminal) domain at the carboxyl terminus. While most cancer-predisposing mutations of BRCA1 result in gross truncation of the protein, 5-10% of the disease-associated mutations lead to single amino acid substitutions, many of which occur in the RING or BRCT domains (15). This suggests that both domains are pivotal to BRCA1 function in tumor suppression.

The BRCT domain of BRCA1 is required for BRCA1 function in both transcriptional activation and DNA repair (3-10). When tethered via the GAL4 DNA binding domain (DBD), the BRCT domain is capable of stimulating transcription from a GAL4-responsive reporter gene (7, 8) and remodeling chromatin from chromosomally embedded GAL4 binding sites (11). The same region of BRCA1 is also reported to interact with histone modifying enzymes such as the histone acetyltransferase p300 (16, 17) and the human histone deacetylase HDAC (18). Importantly, cancer-predisposing mutations in this region abolish the ability of BRCA1 to activate transcription, enhance DNA repair efficiency, and remodel chromatin. Thus, BRCA1 may utilize the BRCT domain to increase chromatin accessibility and facilitate multiple chromosomal events.

The BRCT domain, first identified in BRCA1, is an evolutionarily conserved motif found in a large number of proteins that are involved in various aspects of chromosomal events, such as DNA repair, replication, recombination, gene activation and silencing, and checkpoint control (1, 2). The functional proximity of the BRCT superfamily raises the possibility that BRCT domains may

share a common biochemical property, such as transcriptional activation and chromatin remodeling as demonstrated for the BRCA1 BRCT domain. Several recent reports suggest a role of BRCT domains in mediating protein-protein interactions with another BRCT domain or a different structural module. For example, the BRCT domains present in DNA ligase III and XRCC1, two mammalian DNA repair proteins, interact with each other strongly (19). In addition, the BRCT domain of BRCA1 binds CtIP, a transcriptional co-repressor (20-22). Despite these findings, it remains to be determined whether BRCT domains utilize a common biochemical feature to regulate a specific nuclear process.

The BRCT domain is an approximately 100-amino acid long region defined by distinct conserved patches of hydrophobic residues (see the alignment in Figure 3) (1, 2). X-ray crystallographic study of the second BRCT domain of the repair protein XRCC1 shows that it contains a four-stranded parallel β–sheet encircled by three α-helices (23). Based on the XRCC1 BRCT structure, a model for the BRCT domain of BRCA1 has been constructed and a few cancer-predisposing mutations in this region are predicted to either disrupt the interface or cause incorrect folding (23). However, most of the conserved amino acid residues in the BRCA1 BRCT domain have not been associated with familial breast or ovarian cancers (15). Therefore, the relevance of these conserved residues to the BRCA1-mediated transcriptional activation remains to be explored.

In this study, we compared the BRCT domains of human BRCA1 and a number of yeast proteins for their potential to stimulate transcription and induce changes in chromatin structure. Our work shows that the BRCT domain of the *Saccharomyces cerevisiae* RAP1 (scRAP1), but not the other yeast BRCT domains tested, displays in vivo properties similar to those shown for the BRCT domain of BRCA1. Furthermore, mutational study demonstrates that a number of the highly conserved residues in the BRCT domains of scRAP1 and human BRCA1 are important for transcriptional activation by these BRCT domains.

EXPERIMENTAL PROCEDURES

Plasmids and cells For the yeast-based transcription assay shown in Figure 1, the lacZ reporter plasmid carrying two GAL4 binding sites was integrated into the yeast strain RL1 as previously described (24). To construct the reporter plasmid with five GAL4 binding sites used in Figure 5, an XbaI-HindIII fragment containing the five GAL4 sites from the vector G5BCAT (25) was blunt-ended and cloned into the blunt-ended XhoI site in the lacZ reporter construct pJL638 (26). The resulting plasmid was linearized with StuI and integrated at the yeast *URA3* locus. The luciferase reporter plasmid used in human cells contains four GAL4 binding sites in front of the *fos* TATA element as described previously (27).

Site-directed mutagenesis was performed using the Kunkel method as described (28). To construct the yeast expression vectors for the GAL4 fusion proteins, the sequences encoding the following BRCT domains were amplified by a standard PCR method: BRCA1 (aa1560-1863); DPB11(a,b)(aa1-220); DPB11(c,d)(aa322-579); ESC4(e,f)(aa841-1070); RAD9(aa1027-1309); RAP1(aa121-208); and RFC1(aa153-243). The PCR fragments were subsequently cloned into the XbaI and BamHI sites immediately downstream of the HA-GAL4(1-94) sequence in the *CUP1* expression vector described previously (24). The entire sequence of the amplified fragments was verified by sequencing. The resulting expression vectors were integrated into the *LEU2* locus on the yeast chromosome. For expression of the fusion proteins in human cells, the BRCT domain of BRCA1 was cloned into the XbaI-BamH1 sites in the expression vector pCGHA-GAL4(1-94) (27).

The RL1 yeast strain used for the MNase sensitivity assay and the β-galactosidase assay was described previously (24). Human 293T cells, generously provided by T. Ouchi at the Mount Sinai School of Medicine, were maintained in DMEM medium with 10% fetal calf serum.

Immunoblotting and MNase Assay These assays were performed as described previously (24), except that all liquid growth media contained 100 μM copper sulfate for induction of the GAL4 derivatives. The immunoblots were probed with an anti-HA antibody (12CA5).

Transcription Assays The yeast transcription assay was performed and the specific activity was calculated following a standard protocol (29). For the luciferase assay, human 293T cells were transfected using Lipofectamine 2000 from Gibco/BRL. The following plasmids were included in the transfection: the luciferase reporter (0.5 μg), the β-galactosidase reporter (0.5 μg), and the appropriate GAL4 fusion expression plasmid (1 μg). Transfected cells were harvested forty hours later and the luciferase and β-galactosidase activities were determined following the procedures previously described (27). The β-galactosidase activity was used as an internal control for transfection efficiency.

RESULTS AND DISCUSSION

The scRAP1 BRCT Domain is a Transcription Activation Domain---To test the possibility that BRCT domains besides that of BRCA1 may also be capable of activating transcription and remodeling chromatin, we fused the hemagglutinin (HA)-tagged GAL4-DBD to the BRCT domains of several yeast proteins (Figure 1a). The BRCT-containing proteins chosen here are known for their roles in regulation of various aspects of chromosomal events. These include Dpb11p involved in DNA replication and S-phase checkpoint (30); Rfc1p in DNA replication and repair (31); Rad9p in DNA damage checkpoint (32); Rap1p in transcriptional activation, silencing, and telomere length maintenance (33, 34); and Esc4p in gene silencing (1). As shown in Figure 1b, all GAL4 fusion proteins were expressed with the expected sizes and at a comparable level in yeast.

The potentials of these fusion proteins to activate transcription were analyzed using a GAL4-responsive lacZ reporter gene (Figure 1c). Consistent with previous findings(7, 8), GAL4-

BRCA1 activated transcription in yeast as well as human cells (compare column 1 and 2 in Figure 1c; also see Figure 4). Of all the other BRCT fusion proteins tested, only GAL4-RAP1 gave rise to an elevated β-galactosidase activity (compare column 1 with 7), albeit to a lesser extent than GAL4-BRCA1. None of the remaining GAL4-BRCT constructs showed any appreciable levels of transcriptional stimulation (columns 3-6, and 8). While it remains possible that the lack of transcription activation may be due to improper folding of the BRCT domains in the GAL4 fusion context, our data suggest that at least a subset of the BRCT domains can act as transcriptional activation domains.

The scRAP1 BRCT Domain can Remodel Chromatin---To explore the mechanisms by which the scRAP1 BRCT domain activated transcription, we examined the effect of the GAL4 derivatives on chromatin structure. The yeast strain used in this study contains five GAL4 binding sites engineered near the autonomously replicating sequence 1(ARS1) on chromosome IV (24). It has been shown that, while all GAL4 derivatives can get access to the chromosomally embedded GAL4 binding sites, only those that activate transcription and chromosomal replication are capable of inducing distinct changes in the micrococcal nuclease (MNase) digestion pattern around the GAL4 binding sites (11). As shown in Figure 2, tethering of the BRCT domain of BRCA1 to the chromosome changed the nuclease digestion pattern around the GAL4 binding sites, namely, the intensity of band A was attenuated whereas that of band B enhanced (indicated by arrows; compare lanes 1-2 with 3-4). The same change was observed with GAL4-RAP1 (lanes 13 and 14), but not with any of the transcriptionally inactive GAL4-BRCT fusion proteins (lanes 5-12, 15-16). Thus, transcriptional activation by the BRCT domains correlates with their ability to induce changes in chromatin structure.

The observed changes in chromatin structure are unlikely due to presence of the entire transcription machinery or initiation of transcription *per se* in the vicinity of the GAL4 sites, as the GAL4 binding sites are located at least 800 bp away from the nearest transcriptional promoter.

Rather, a chromatin remodeling complex may be recruited by the scRAP1 BRCT domain to induce the changes in the local chromatin structure. In keeping with this notion, the BRCT domain of BRCA1 has been shown to associate with certain histone modifying enzymes such as the histone acetyltransferase p300 (16, 17) and the human histone deacetylase HDAC (18). Taken together, the transcription activation and chromatin remodeling assays may provide useful tools for identifying additional BRCT domains that share the same biochemical features as those of scRAP1 and BRCA1.

The Conserved Residues in the BRCA1 BRCT Domain are Important for Transcriptional Activation--- To address the significance of the conserved residues in transcriptional activation, we introduced mutations in several conserved blocks of the second BRCT domain of BRCA1 (HsBRCA1-b in Figure 3). These include the N-terminal tail, α1, α3, and the C-terminal tail as predicted based on the structure of the XRCC1 BRCT domain (23). The four-stranded β sheet and α2 were not targeted for mutagenesis in this study, as the β sheet is predicted to form the core of the structure and the corresponding mutant proteins tend to be unstable (data not shown), and the sequence for α2 is not universally present in the BRCT superfamily.

A total of eleven alanine substitution mutants were constructed in the context of the GAL4-BRCA1 fusion. To analyze the mutational effects in a more physiological context, the wild type and mutant fusion proteins were expressed and their transcriptional activity was assessed in human 293T cells (Figure 4a and b). As expected, the wild-type GAL4-BRCA1 fusion can potently stimulate transcription from a GAL4-responsive luciferase reporter construct (compare lane 2 with 1 in Figure 4b). Mutations at multiple conserved amino acid residues either reduced or completely abolished the transcriptional activity. For example, IF1760-1AA of the N-terminal tail, L1780A and MV1783-4AA of α1, G1788A between α1 and β2, VL1838-9AA and S1841A of α3, and YLI1853-5AAA of the C-terminal tail all severely impaired the *trans*-activation capability of the BRCT domain (compare lane 2 with lanes 3, 6, 8, 9, 10, 11 and 13 in Figure 4b). Of all the

conserved residues mutated in this study, only L1764A failed to affect BRCT function in a significant manner (lane 4 in Figure 4b). In addition, mutations of three non-conserved residues (D1778A, W1782A, and Q1848A) did not cause deleterious effects either (lanes 5, 7, and 12), despite the fact that the corresponding residues of D1778 and W1782 in XRCC1 are involved in dimer formation between two BRCT domains (23). The results are also summarized with signs below the sequence of the BRCT domain in Figure 3.

Natural mutations at M1783, G1788, and S1841 have been identified as unclassified mutant variants in terms of cancer predisposition (15) and predicted to affect protein folding (23). The alanine substitution mutants at these three positions indeed abrogated the transcriptional activation by GAL4-BRCA1 (lanes 8, 9, and 11 in Figure 4b), suggesting that the corresponding natural mutations at these sites may represent genuine cancer-predisposing mutations. Taken together, the data strongly suggest that a majority of the conserved amino acid residues in the BRCT domain are critical for BRCA1 function in transcriptional activation. Our results in human cells are also in agreement with a recent yeast-based study of the BRCA1 BRCT domain (35). In particular, the three regions mutagenized in both studies (F1761, L1780, and YLI1853-5) are important for *trans*-activation in human as well as yeast cells.

Evidence for an Inhibitory Region in the scRAP1 BRCT Domain---Next, we asked whether the conserved residues in the scRAP1 BRCT domain are important for transcriptional activation as well. In addition to the BRCT domain in the N-terminus of scRAP1, the protein also contains several other domains, such as the central Myb-like DNA binding domain and the *trans*-activation and silencing domains in the C-terminus (36). A human homologue of scRAP1 (hRAP1) was recently identified (37). While hRAP1 is highly diverged from its counterparts in *Scaccharomyces cerevisiae* (scRAP1) and *Kluyveromyces lactis* (klRAP1), the BRCT domain, the Myb-related motif, and the silencing domain are conserved (see alignment of the BRCT domain in Figure 3). Interestingly, the potent *trans*-activation domain identified in scRAP1 and klRAP1 is not present as

such in hRAP1 (37). To ascertain the relevance of the BRCT motif to the transcriptional activation conferred by GAL4-RAP1, we changed several amino acid residues in the regions that correspond to those in the BRCA1 BRCT domain studied above. As illustrated in Figure 3, alanine substitutions were introduced at PL125-6 of the N-terminus; L143, N144, L149, R151, and LI152-3 of α1; GG157-8 between α1 and β2; Y190, IK191-2, and C194 of α3; and YLV205-8 of the C-terminal tail.

As shown in Figure 5, two mutations in α3 (Y190A and C194A) and one in the C-terminal tail (YLV205-8AAA) either reduced or completely abolished the ability of GAL4-RAP1 to stimulate transcription (lanes 5, 6 and 7). Notably, the corresponding residues in the BRCA1 BRCT domain had a similar deleterious effect on transcriptional activation (see W1837R in ref. 11; and S1841A and YLI1853-5AAA in Figure 4). Therefore, the conserved residues toward the end of both BRCT domains are important for the transcriptional activation function. However, mutations at several other conserved positions of the scRAP1 BRCT domain yielded a quite unexpected result. Instead of impairing the function of the BRCT domain, these mutations significantly increased the transcriptional activity of GAL4-RAP1 (compare lane 8 with 9-13 of Figure 5; note the two different scales used for lanes 1-7 and lanes 8-13). Most strikingly, PL125-6AA of the N-terminus, LI152-3AA of α1, and GG157-8AA of the loop between α1 and β2 were at least 15 fold as robust as the wild-type protein (compare lane 8 with 9, 11, and 12 of Figure 5). Thus, these conserved residues may constitute a region that negatively modulates the BRCT function. This feature seems to be unique to the BRCT domain of scRAP1, as mutations at the corresponding residues in the BRCT domain of BRCA1 had the opposite effect on transcriptional activation in human cells (Figure 4). The mutational results are also summarized with signs above the sequence of the scRAP1 BRCT domain in Figure 3.

Based on the crystal structure of the XRCC1 BRCT domain (23), the residues involved in repressing the BRCT function in scRAP1 (PL125-6, L149, LI152-3, GG157-8, and IK191-2) are

predicted to reside proximally in the tertiary structure. Moreover, L149 and LI152-3 of α1 and IK191-2 of α3 are likely to be involved in mediating the intra-molecular interaction between α1 and α3, whereas the highly conserved Gly-Gly residues between α1 and β2 may be important for proper orientation of the two helices. Thus, it is conceivable that the interaction between α1 and α3 may result in a conformation that is unfavorable for transcriptional activation. Alternatively, the amino acid residues of interest may be involved in an interaction with a transcriptional repressor. In either situation, the negative regulatory region of the scRAP1 BRCT domain may serve as a molecular switch that coordinates the multiple functions of RAP1 in transcriptional activation, gene silencing (33), and telomere length maintenance (38), etc.

The BRCT domain is identified by sequence comparison, yet its role in the biological function of the full-length protein remains obscure. In fact, the entire N-terminal sequence including the BRCT domain is not essential for scRAP1 to support cell viability (39). In this regard, it is somewhat puzzling that the BRCT domain is well conserved between yeast and human, whereas the sequence for the potent *trans*-activation domain found in scRAP1 and klRAP1 is not (37). While the exact contribution of the BRCT domain to RAP1 function awaits further investigation, it is possible that the transcriptional activation and chromatin remodeling property associated with the BRCT domain may be important for a more specialized, non-essential function of scRAP1. It is also tempting to speculate that, in the absence of a strong *trans*-activation domain in hRAP1, the BRCT domain of the human RAP1 may play a more prominent role in RAP1-mediated regulation of chromosomal events in human cells than its yeast counterparts.

Acknowledgments

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FOOTNOTES

The abbreviations used are: BRCT, BRCA1 C-terminal; MNase, micrococcal nuclease; HA, hemagglutinin; DBD, DNA binding domain; ARS, autonomously replicating sequence.

FIGURE LEGENDS

Figure 1. Activation of transcription by various GAL4-BRCT fusion proteins. **a.** Diagram illustrating the relative position of the BRCT domains in BRCA1 and the yeast proteins. Each shaded region represents one BRCT motif. The regions marked by brackets are included in the same GAL4 fusion protein. **b.** The HA-tagged GAL4 derivatives were detected by immunoblotting of an equal amount of whole cell extracts. The molecular weight markers are indicated on the left. Asterisks indicate the bands representing the full-length fusion proteins. See the text for more description of each fusion protein. **c.** The β-galactosidase activity from cells that express various GAL4-BRCT fusion proteins. The results shown are an average of data from at least four independent experiments.

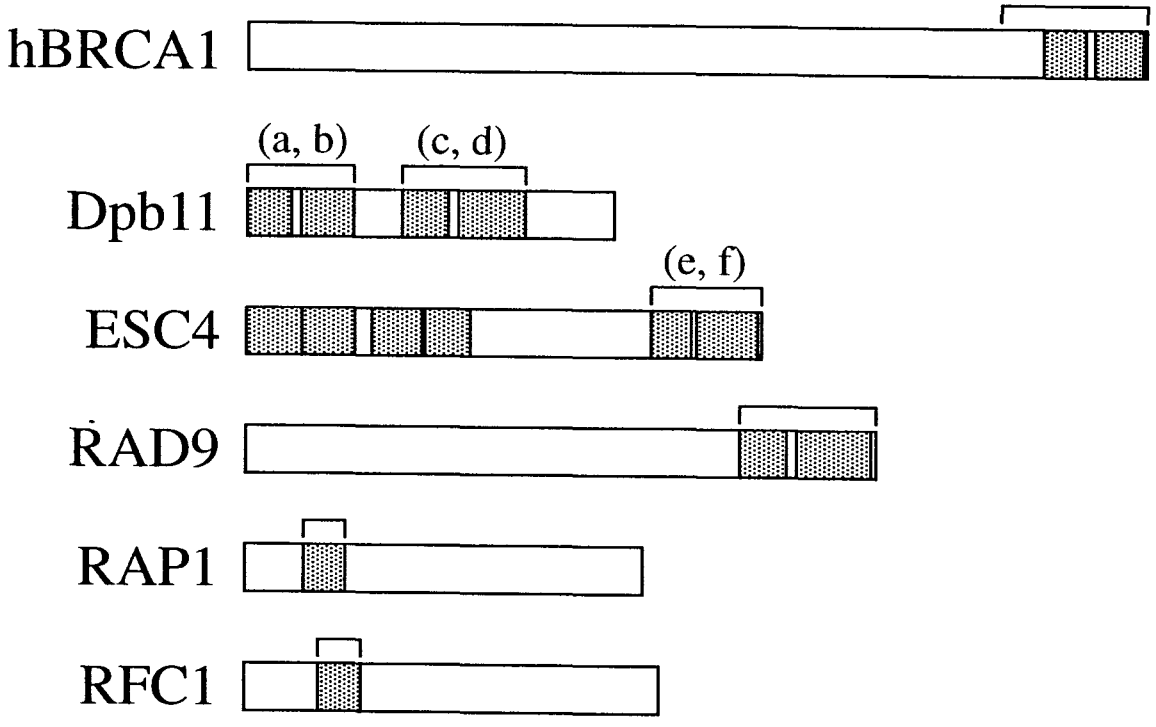
Figure 2. The BRCT domains from BRCA1 and scRAP1 can induce changes of the chromatin structure *in vivo*. Indirect end-labeling assay was used for the analysis of the MNase digestion pattern around the chromosomally embedded GAL4 binding sites. Two time points of the MNase treatment are shown for each GAL4 derivative. The EcoR1 fragment containing the *ARS1* locus

was revealed by a radioactive probe derived from an NheI-EcoR1 restriction fragment (indicated by a thick bar on the left). The arrows (A and B) indicate the two bands the intensities of which were most significantly affected by GAL4-BRCA1 and GAL-RAP1. The approximate positions of the GAL4 binding sites and the four *cis*-elements of *ARS1* are indicated on the left.

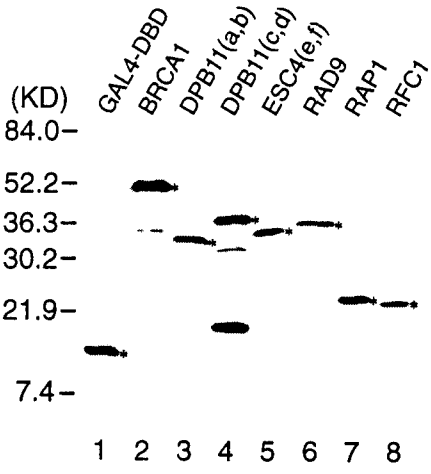
Figure 3. Diagram showing the primary sequence and the predicted secondary structure of the BRCT domains. The highly conserved amino acid residues are highlighted in bold and shade, and the similar residues in bold only. The numbers on the top and bottom of the sequences correspond to the positions of amino acids in scRAP1 and hBRCA1, respectively. Asterisks and triangles indicate those amino acids that are mutated in this study. Asterisks are those that show no obvious phenotypes. Triangles pointing up designate mutants that cause "super-activation", and those pointing down indicate mutations that reduce transcriptional activity.

Figure 4. Mutational analysis of the second BRCT domain of BRCA1 in a human cell-based transcription assay. **a.** Expression of the HA-tagged GAL4 –BRCA1 chimeras in human 293T cells. The immunoblot is probed with an anti-HA antibody (12CA5). **b.** Luciferase assays are performed using cell lysates from human 293T cells transfected with a luciferase reporter, a β-galactosidase (as an internal control) reporter, and the GAL4-BRCA1 expression vectors. The transcriptional activity is expressed as fold of activation.

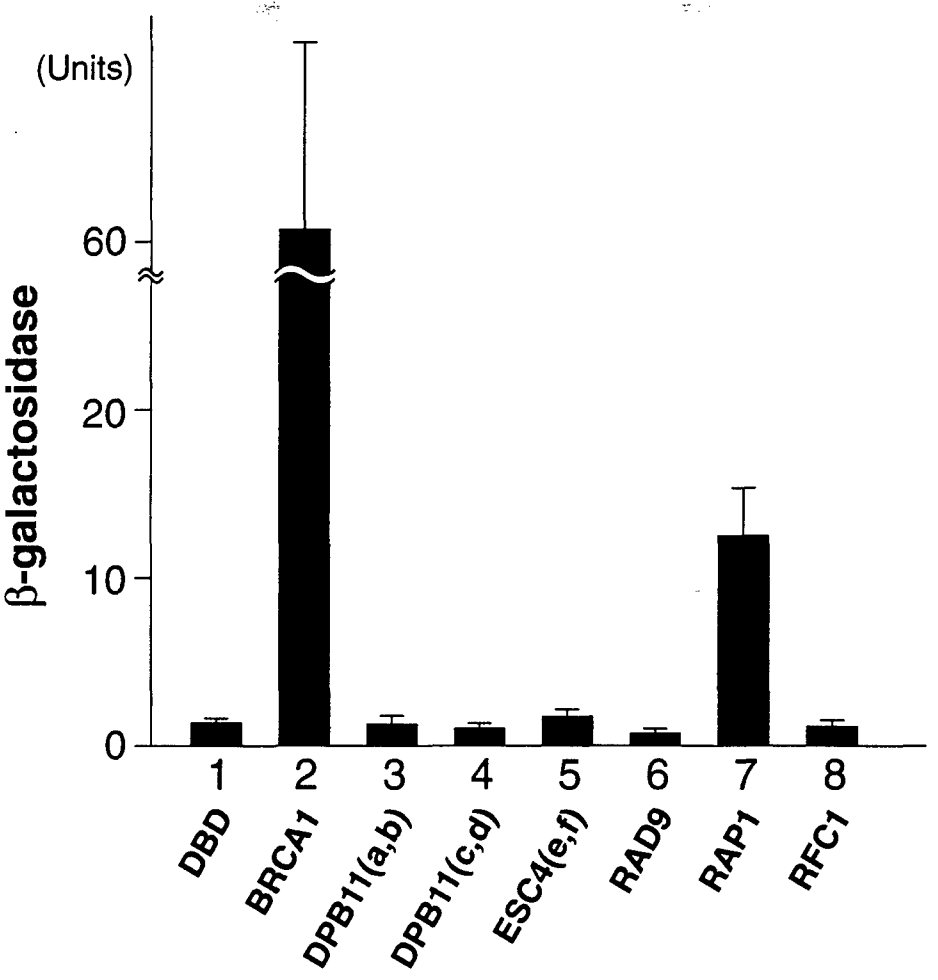
Figure 5. Mutational analysis of the scRAP1 BRCT domain using a yeast-based transcription assay. Liquid β-galactosidase assays are carried out using lysates from yeast cells that express various GAL4 derivatives. For a convenient comparison, β-galactosidase values for the GAL4-derivatives are shown in two different scales (left and right panels). The wild-type activity is set as 100%.



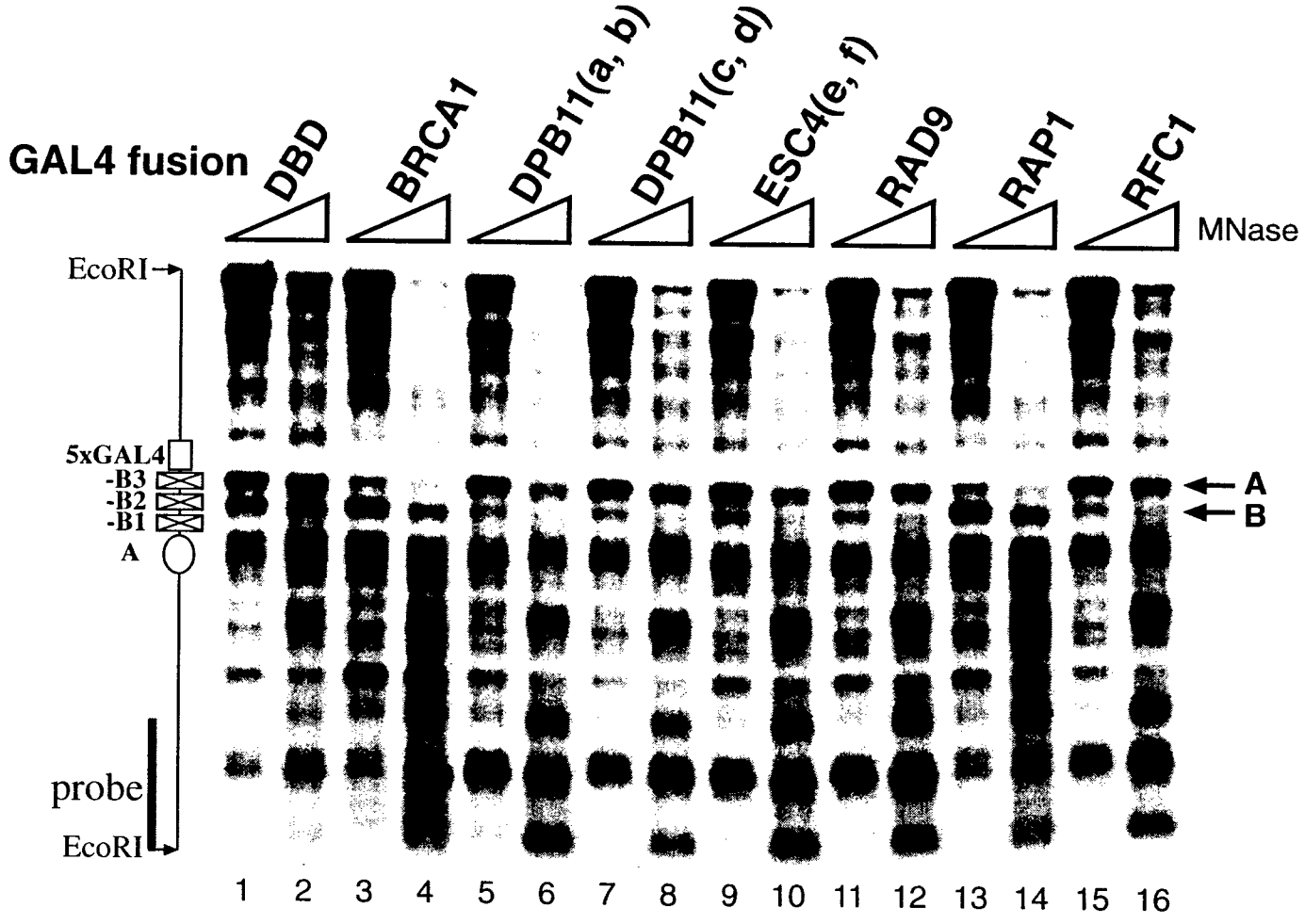
Miyake T., et al Fig.1a



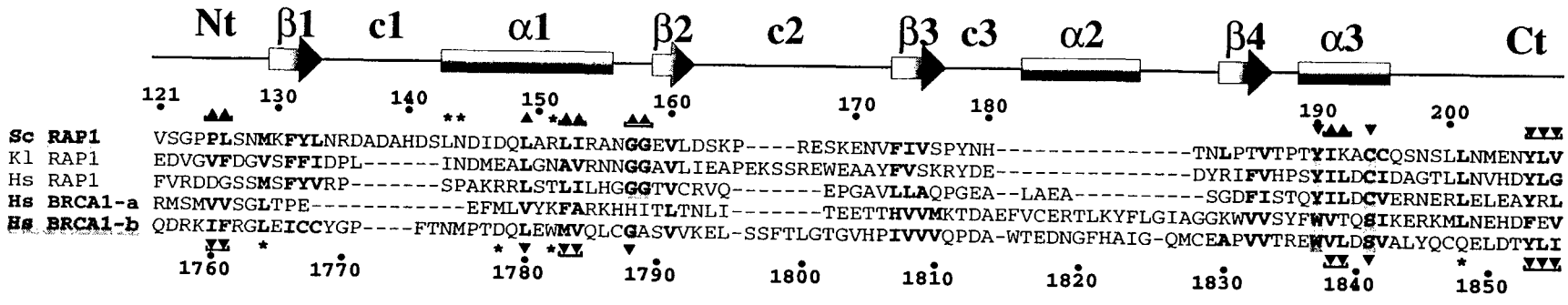
Miyake T., et al Fig.1b



Miyake T., et al Fig.1c



Miyake T., et al Fig.2



Miyake T., et al Fig.3

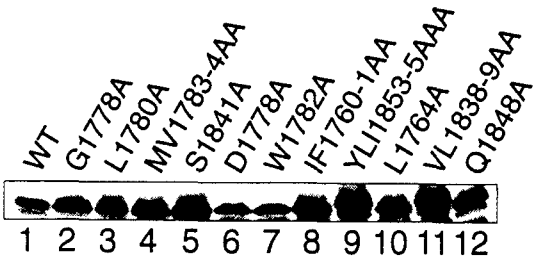
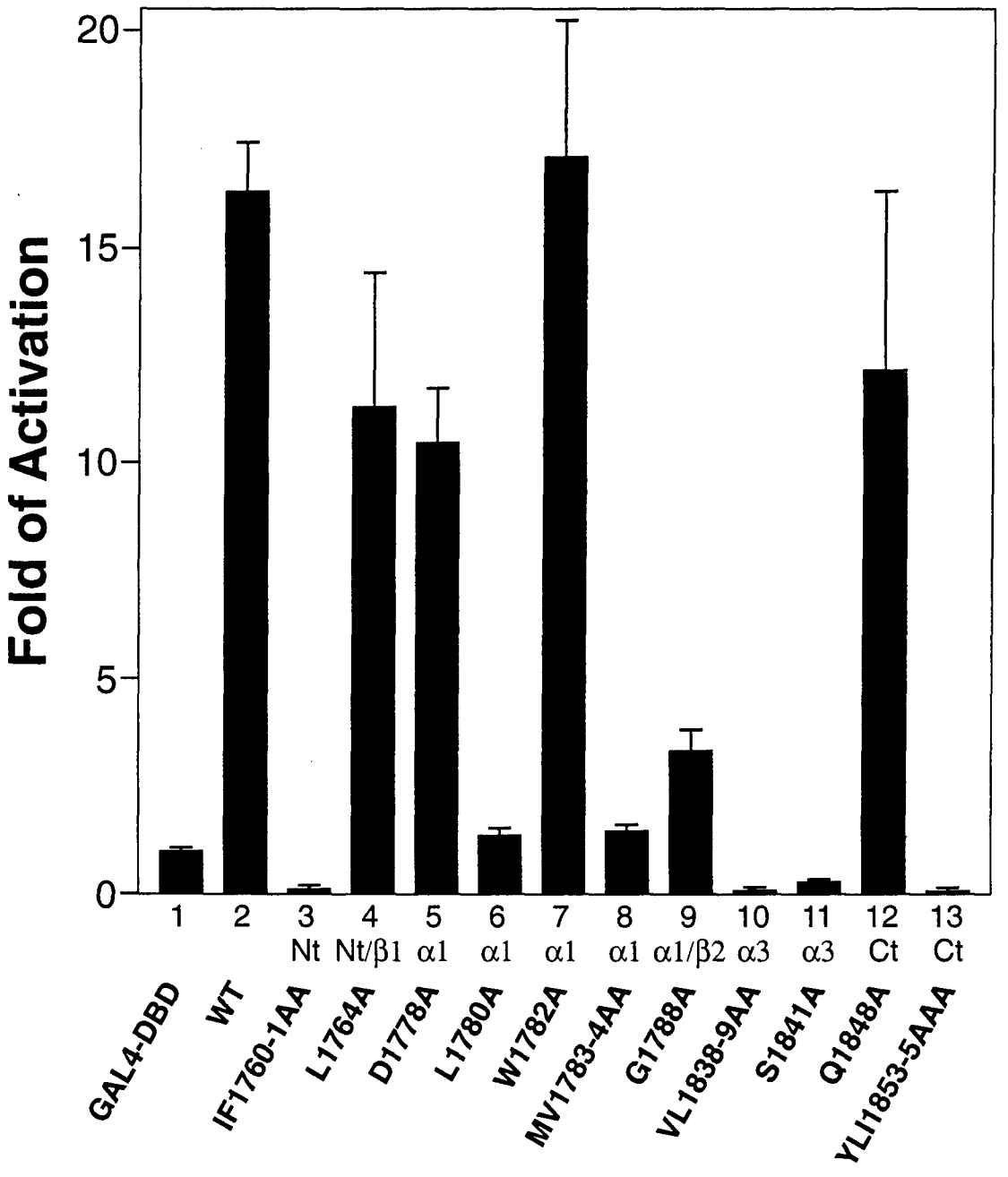
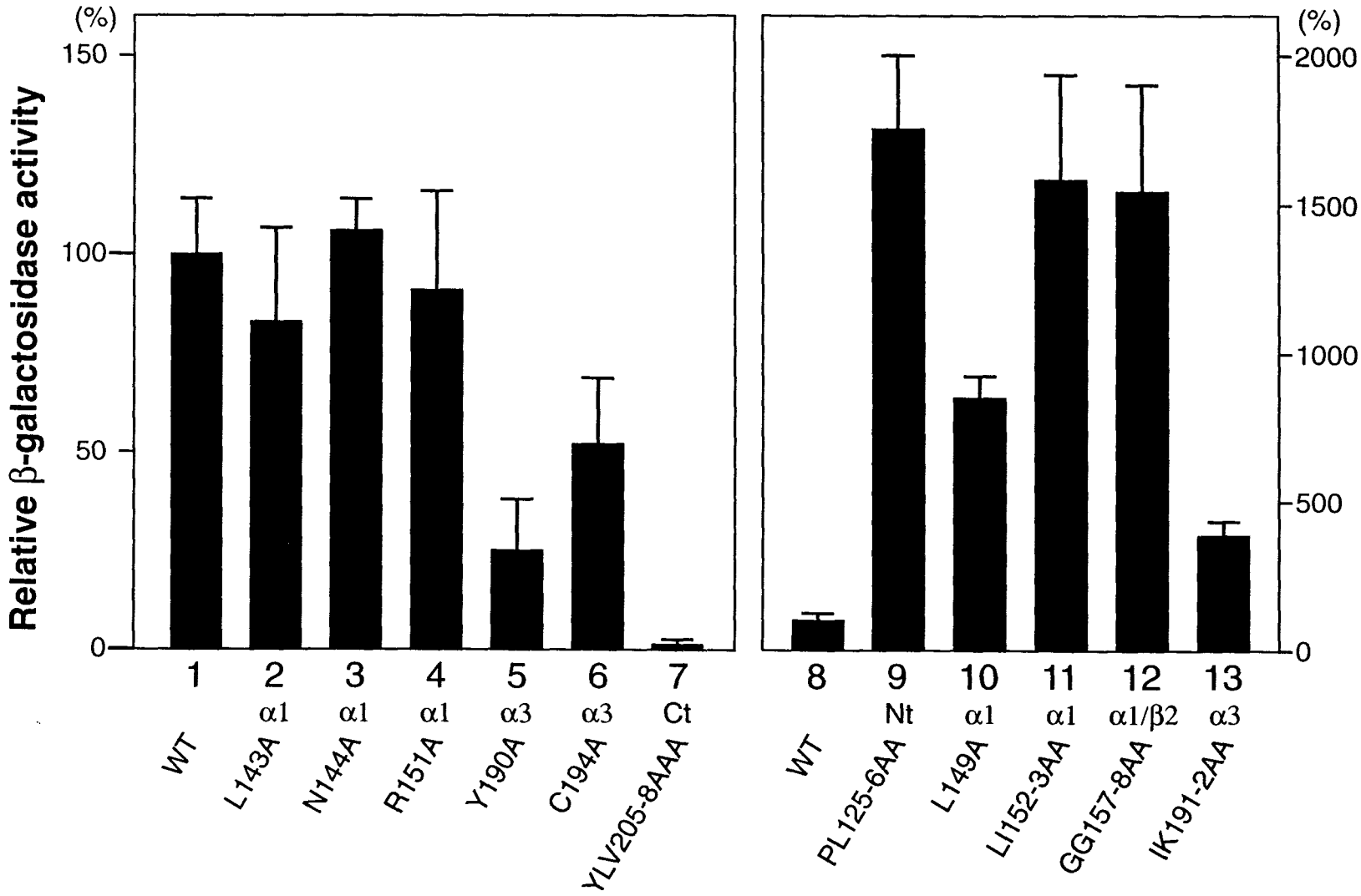


Fig. 4a Miyake T., et al



Miyake T., et al Fig.4b



Miyake T., et al Fig.5

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REPLY TO
ATTENTION OF

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