The Dbh homology (DH) domain of BCR in P210BCR-ABL (P210/WT) has been thought to have a negative effect on the activation of BCR-ABL because P185BCR-ABL, in which this region is physically deleted, has stronger biochemical and biological activities. To study the role of the DH domain of BCR in the background of P210/WT, the region was replaced with homologous sequences derived from Dbl (P210/Dbl) or CDC24 (P210/CDC24) or with irrelevant sequence from LacZ (P210/LacZ) or luciferase (P210/Luci). Surprisingly, the abilities to transform Rat 1 cells or mouse bone marrow cells and induce growth factor independence in interleukin 3-dependent mouse Ba/F3 cells were retained only in P210/Dbl. However, even P210/Dbl could not achieve the wild type level of surviving potential against genotoxins in Rat 1 cells and in Ba/F3 cells. Activation of Akt correlated with the biological changes in Rat 1 cells but did not correlate with the biological changes in Ba/F3 cells. The DH domain was not tyrosine-phosphorylated in vitro, nor could we find any differences in peptide mapping between in vitro phosphorylated P210/WT and P210/Dbl. Although functions of the DH domain remain to be discovered, we propose that the DH domain makes positive contributions to P210BCR-ABL.

The oncprotein BCR-ABL is generated on the Philadelphia chromosome found in two types of human leukemias, chronic myeloid leukemia (CML) and acute lymphocytic leukemia (1). Clinical manifestation of CML is unique. Massive expansion of myeloid cells of every maturation stage, which is observed in chronic phase, is inevitably followed by an acute leukemic phase called blast crisis. P210BCR-ABL found in CML and P185BCR-ABL in acute lymphocytic leukemia differ in the BCR sequence that participates in the formation of BCR-ABL (1, 2). The BCR sequence that is missing in P185 but retained in P210 has a homology to the GDP-GTP exchanger domain of Dbl and CDC24 and is therefore called the Dbl homology (DH) domain (3). P185BCR-ABL has a stronger tyrosine kinase activity than P210BCR-ABL, and this correlates well with biological phenotypes (2). P185BCR-ABL has stronger transforming potentials for Rat 1 fibroblasts and mouse bone marrow cells than P210BCR-ABL. In a transgenic animal model, some P210BCR-ABL animals are even free of disease (4). Because the sequence encoded by the BCR first exon plays a pivotal role in the activation of BCR-ABL by binding to the ABL SH2 domain, it has been suggested that the DH domain is simply an inhibitory spacer (5, 6). The difference in the transforming potential cannot explain the clinical phenotype of CML, and functions of the DH domain in P210BCR-ABL have been poorly understood.

Actin cytoskeleton staining experiments suggested that disruption of F-actin was observed in a P185-equivalent form of BCR-ABL but not in P210-expressing Rat 1 cells, which implies that the DH domain plays a role in stabilization of actin filaments (7). We could never show GDP-GTP exchange activities associated with this region but a report claimed that it activates CDC42, RhoA, and Rac (8, 9). If this is the case, P210 could elicit signals via Rho family proteins. Those small GTP-binding proteins are involved not only in the activation of cytoskeleton but also in cell growth and gene expression (10, 11). For example, cellular transformation by Dbl is mediated by the activation of its substrates, Rho-like GTPases including CDC42 (12). Transformation by BCR-ABL has been reported to be blocked by dominant-negative Rac (13). BCR-ABL has also been documented to preferentially activate Jun kinase to mitogen-activated protein kinase (14). Although Ras is activated by BCR-ABL via Grb-2 and Shc, there are Ras-dependent and -independent pathways leading to the activation of Jun kinase, and CDC42 was shown to mediate Jun kinase activation (15–19).

Recently, we have reported that P210BCR-ABL but not P185BCR-ABL binds to the xeroderma pigmentosum group B protein (XPB) through the DH domain and inactivates its DNA unwinding activity, at least in vitro (20). Because XPB is an essential component of the basal transcription factor TFIIH involved in DNA repair and transcription, we have hypothesized that the blast crisis observed in CML could be caused by a defect in DNA repair that potentially induces genomic instability. We have also shown that XPB does not bind to other DH domain-containing proteins such as CDC24 and Dbl (21).

All of this information suggests that the DH domain of BCR may not be a simple spacing region in P210BCR-ABL but could be of structural as well as functional importance. To further address this issue, here we report biochemical and biological analyses of replacement mutants of this region.

EXPERIMENTAL PROCEDURES

Molecular Construction—The BCR fragment BglII (nucleotide 1704) to BstII (nucleotide 2809) encoding the Dbl homology domain (amino acids 413–789) was replaced with homologous sequences in Dbl or yeast CDC24 or with irrelevant sequences of similar length from LacZ or luciferase (21–24). Primers used for polymerase chain reactions are 5′-GGAAGATCTGCGAAGACACTTTGACATT3′ and 5′-GGAAGATCTTGAATATGAGGATGATGAT-3′ for CDC24 and Dbl (21).

The Journal of Biological Chemistry Vol. 276, No. 42, Issue of October 19, pp. 39462–39468, 2001
This paper is available on line at http://www.jbc.org
5'-GAAGATCTTCTGGTCCGGAAGTC-3' and 5'-GAAGATCTGAA-
ACTATCCCTCTTG-3' for Dbl (amino acids 183–422), 5'-GAAGAT-
CTTATGCGGTGGTG-3' and 5'-GAAGATCTCTGAAAGTTGG-
GGGTGGTG-3' for luciferase, and 5'-GAAGATCTGTGCCTGGAGA-
A-3' and 5'-GAAGATCTGACGAGTGTACGTCGCC-3' for LacZ. Prim-
ers were designed to create BglII sites at both ends of all fragments
generated by polymerase chain reaction. Noncoding frames of the or-
iginal LacZ and luciferase sequences were integrated into the P210BCR-
ABL sequence in-frame.

**MOLECULAR REAGENTS AND ANALYSES**—Anti-BCR antibody (Ab) was pur-
chased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-ABL Ab was
obtained from Upstate Biotechnology (Lake Placid, NY), anti-phos-
photyrosine Ab PY20 was obtained from ICN Biomedicals, and anti-Akt and
anti-phospho-Akt (Ser-473) were obtained from New England Bio-
labs. Anti-Vav, Anti-Crkl, and anti-Shc Abs were obtained from Upstate
Biotechnology, anti-STATS and anti-ABL Abs were obtained from Santa
Cruz Biotechnology, and anti-actin Ab was obtained from Chemicon.
Methyl methanesulfonate (MMS), cytosine arabinoside, and cisplatin
were purchased from Sigma. Western blot analysis, immunoprecipita-
tion, in vitro kinase assay, and peptide mapping of the replaced region
were performed independently three times. P210BCR-ABL transformants
were observed only in P210/Dbl, a P210/BCR-ABL mutant in
which the DH region was replaced with the sequence from Dbl
(Fig. 2, B, D, and E). Recruitment of Grb-2 (18) was found only
in the wild type (P210/WT) and P210/Dbl (data not shown).
When immunoprecipitated P210 and its mutants were sub-
jected to an in vitro kinase assay with enolase as a substrate,
there was almost no difference in phosphotransferase activity
(Fig. 2, F and G) as well as in autokinase activity (data not shown).
As expected, transformation of Rat1 fibroblasts corre-
lated with the in vivo kinase activity (Fig. 3, A and B). Inter-
estingly, there was a clear difference in the morphology of cells
transformed by P210/WT and P210/Dbl (Fig. 3C). Both of these
could transform mouse bone marrow cells (Table I).

The physical deletion of the DH domain creates P185BCR-
ABL, which is more activated or autophosphorylated than
P210BCR-ABL (2), and this information strongly suggests that
there may be a structure-based essential difference between
P210 and P210/Dbl (percentage of survival, 2.4
6.5%) in survival exper-
iments performed independently three times. P210BCR-ABL has
been shown to activate phosphatidylinositol 3-kinase
(PI3K), which is involved in transformation as well as anti-
apoptosis (28). Activation of Akt, a downstream effector of
PI3K, was observed in Rat1 cells that expressed P210/WT but
not P210/Dbl (Fig. 4, A–C).

Combined with the results in Figs. 2 and 3, these data
indicate that even the homologous sequences from CDC24 or
Dbl cannot fully substitute for the DH domain of BCR in
P210BCR-ABL when expressed in Rat1 cells. A fragment of Dbl
that was used for replacement (amino acids 466–832) includes

dissociation of kinase activity in vitro and in vivo—To
study the functional significance of the DH domain in
P210BCR-ABL, we have undertaken a strategy to replace the
region with homologous sequences such as those in CDC24 and
Dbl (21% and 21% identity over 236 amino acids, respectively)
or with nonhomologous or irrelevant sequences from luciferase
and lacZ (Fig. 1). We thought that polypeptide sequences of
approximately the same length would serve as suitable substitu-
tions for the DH domain of BCR without eliminating a struc-
turally positive contribution, if any, of this region in P210BCR-
ABL. The replaced region contains the entire DH domain, but
a part of the PH domain of BCR was also replaced with other
sequences.

The wild type and the DH domain mutants were retrovirally
expressed in Rat1 fibroblasts at a roughly equal level (Fig. 2, A
and C). Surprisingly, the wild type level of autophosphorylation
and transphosphorylation of an in vivo substrate (p62Dok) (27)
were observed only in P210/Dbl, a P210/BCR-ABL mutant in
which the DH region was replaced with the sequence from Dbl
(Fig. 2, B, D, and E). Recruitment of Grb-2 (18) was found only
in the wild type (P210/WT) and P210/Dbl (data not shown).
When immunoprecipitated P210 and its mutants were sub-
jected to an in vitro kinase assay with enolase as a substrate,
there was almost no difference in phosphotransferase activity
(Fig. 2, F and G) as well as in autokinase activity (data not
shown). As expected, transformation of Rat1 fibroblasts corre-
lated with the in vivo kinase activity (Fig. 3, A and B). Inter-
estingly, there was a clear difference in the morphology of cells
transformed by P210/WT and P210/Dbl (Fig. 3C). Both of these
could transform mouse bone marrow cells (Table I).

**RESULTS**

Dissociation of Kinase Activity in Vitro and in Vivo—To
study the functional significance of the DH domain in
P210BCR-ABL, we have undertaken a strategy to replace the
region with homologous sequences such as those in CDC24 and
Dbl (21% and 21% identity over 236 amino acids, respectively)
or with nonhomologous or irrelevant sequences from luciferase
and lacZ (Fig. 1). We thought that polypeptide sequences of
approximately the same length would serve as suitable substitutions for the DH domain of BCR without eliminating a structurally positive contribution, if any, of this region in P210BCR-ABL. The replaced region contains the entire DH domain, but a part of the PH domain of BCR was also replaced with other sequences.

The wild type and the DH domain mutants were retrovirally expressed in Rat1 fibroblasts at a roughly equal level (Fig. 2, A and C). Surprisingly, the wild type level of autophosphorylation and transphosphorylation of an in vivo substrate (p62Dok) (27) were observed only in P210/Dbl, a P210/BCR-ABL mutant in which the DH region was replaced with the sequence from Dbl (Fig. 2, B, D, and E). Recruitment of Grb-2 (18) was found only in the wild type (P210/WT) and P210/Dbl (data not shown). When immunoprecipitated P210 and its mutants were subjected to an in vitro kinase assay with enolase as a substrate, there was almost no difference in phosphotransferase activity (Fig. 2, F and G) as well as in autokinase activity (data not shown). As expected, transformation of Rat1 fibroblasts correlated with the in vivo kinase activity (Fig. 3, A and B). Interestingly, there was a clear difference in the morphology of cells transformed by P210/WT and P210/Dbl (Fig. 3C). Both of these could transform mouse bone marrow cells (Table I).

The physical deletion of the DH domain creates P185BCR-ABL, which is more activated or autophosphorylated than P210BCR-ABL (2), and this information strongly suggests that there may be a structure-based essential difference between P210 and P210/Dbl (percentage of survival, 2.4–6.5%) in survival experiments performed independently three times. P210BCR-ABL has been shown to activate phosphatidylinositol 3-kinase (PI3K), which is involved in transformation as well as anti-apoptosis (28). Activation of Akt, a downstream effector of PI3K, was observed in Rat1 cells that expressed P210/WT but not P210/Dbl (Fig. 4, A–C).

Combined with the results in Figs. 2 and 3, these data indicate that even the homologous sequences from CDC24 or Dbl cannot fully substitute for the DH domain of BCR in P210BCR-ABL when expressed in Rat1 cells. A fragment of Dbl that was used for replacement (amino acids 466–832) includes
the GDP-GTP exchanger domain (amino acids 498–737) that has been reported to show GDP dissociation activity, at least in vitro, and the PH domain (amino acids 708–812) (9). Therefore, the activities of P210/Dbl may represent either structurally satisfactory substitution for the DH domain of BCR by the corresponding Dbl sequence to activate ABL or the exchanger activity of Dbl or both. However, the dissociation of biological and biochemical potential between P210/WT and P210/Dbl shown in survival assays against MMS indicates that there may be a function of BCR in the DH domain that cannot be replaced by the Dbl sequence.

**Structural Significance**—BCR-ABL has been shown to induce growth factor independence in many cytokine-dependent hematopoietic cells (29). Interleukin (IL)-3-dependent mouse Ba/F3 cells were infected with retroviruses expressing P210/ABL or its mutants and were selected by G418 in the presence of IL-3. Those populations of cells expressed BCR-ABL or its mutant proteins in roughly equal amounts (data not shown). When those cells were deprived of both serum and IL-3, cells expressing P210/CDC24, P210/Luci, or P210/LacZ showed poor survival (Fig. 5A). Ninety-six-well assays in culture medium containing serum but not IL-3 showed a high efficiency (>90%) of outgrowth of IL-3-independent Ba/F3 cells

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**Fig. 2. Dissociation of kinase activity in vivo and in vitro.** Total lysates (A and B) or anti-ABL immunoprecipitates (C and D) from Rat1 cells expressing mock (vector alone; lane 1), P210/ABL (P210/WT; lane 2), and the set of the DH domain mutants shown in Fig. 1 including P210/CDC24 (lane 3), P210/Dbl (lane 4), P210/Luci (lane 5), and P210/LacZ (lane 6) were subjected to anti-ABL (A and C) and anti-phosphotyrosine (anti pTyr) Western blotting. Densitometrically scored intensities of signals of phosphotyrosine-containing BCR-ABLS from D relative to those of protein amount in C were calculated and are shown in E. Anti-ABL immunoprecipitates shown in C were subjected to an in vitro kinase assay in the presence of enolase. Phosphorylated enolases were run on a gel and dried, and autoradiography is shown in F. Phosphotransferase activities in F relative to the protein amounts in C were calculated and are shown in G.

**Fig. 3. Transformation of Rat1 cells by the DH domain mutants of P210/ABL.** Soft agar colony assays of the Rat1 cells described in Fig. 2 were performed. Pictures of the colony (A), the relative colony numbers compared with the control P210/WT (B), and the morphology of the Rat1 cells transformed by P210/WT and P210/Dbl (C) are shown.

| Constructs | Bone marrow |
|------------|-------------|
| Mock       | 0/12        |
| P210/WT    | 3/12        |
| P210/CDC24 | 0/12        |
| P210/Dbl   | 1/12        |
| P210/Luci  | 0/12        |
| P210/LacZ  | 0/12        |

**TABLE I**

Transformation of bone marrow cells by BCR-ABL mutants

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The figure shows the dissociation of kinase activity both in vivo and in vitro, with Western blotting and autoradiography. The table lists the transformation of bone marrow cells by BCR-ABL mutants, showing the relative colony numbers and survival efficiencies.
in both P210/WT and P210/Dbl expressers in 7–10 days. In P210/CDC24, the efficiency was 40–50%. However, no wells were found to contain surviving Ba/F3 cells in P210/Luci and P210/LacZ, even at 4 weeks. Therefore, we attempted to obtain cell lines that survived against IL-3 starvation in P210/Luci or P210/LacZ by continuing cultures of the whole infected and G418-selected population of Ba/F3 cells. We expected that the surviving cells would express P210/Luci or P210/LacZ with gain of function mutations or would have up-regulations of other genes in IL-3 signaling independent of the mutant BCR-ABLs. Intriguingly, as shown in Fig. 5, deletions were found in both P210/Luci and P210/LacZ sequences in the Ba/F3 cells that survived for 4–6 weeks. Reverse transcription-polymerase chain reaction analyses revealed an in-frame deletion encompassing the replaced sequence. In P210/Luci, the fusion point on the ABL side was 17 amino acids carboxyl to the ATP-binding motif GXGXGXG (Fig. 5C). Neither immunoprecipitated P210/Luci nor immunoprecipitated P210/LacZ was able to phosphorylate enolase in vitro or bind to Grb-2 (data not shown). Because tyrosine phosphorylation of STAT5 (30) in Ba/F3 cells expressing P210/Dbl-expressing Ba/F3 cells. Given that tyrosine phosphorylation of p62Dok (see Fig. 2B) and Shc (data not shown) also took place in Rat1 cells, it can safely be said that the Dbl sequence is nearly
sufficient to substitute for the DH domain of BCR to activate BCR-ABL without grossly changing the substrate specificity.

We consistently observed a difference between P210/WT and P210/Dbl in sensitivity to cytotoxic agents such as MMS in the background of both Rat1 cells and Ba/F3 cells. Because Akt appeared to be activated in Rat1 cells expressing P210/WT but not P210/Dbl, we measured the kinase activity of immunoprecipitated Akt. However, under conditions in which platelet-derived growth factor could enhance the Akt activity by 2.1-fold over the baseline in IL-3-dependent Ba/F3 cells starved of serum, the levels of Akt activity in Ba/F3 cells expressing P210/WT and P210/Dbl were 1.8- and 2.0-fold, respectively.

**Cytoskeletal Activation**—When the IL-3-independent Ba/F3 cells expressing P210/WT, P210/Dbl, or P210/CDC24 shown in Fig. 5B were subjected to transwell assay, there was a significant difference between P210/WT and P210/CDC24 in the number of cells that migrated through the membrane (Fig. 8A). P210/Dbl showed an intermediate ability for migration. BCR-ABL has been shown to phosphorylate Crkl and Cbl, both of which play an important role in migration of blood cells (33, 34). Tyrosine phosphorylation of both Crkl and Cbl was observed in Ba/F3 cells expressing P210/WT and P210/Dbl but not P210/CDC24 (Fig. 8C), which correlates well with the biological data in Fig. 8A. The wound healing assay also revealed a higher migrating ability in Rat1 fibroblasts that express P210/WT or P210/Dbl than in cells expressing mock (vector only). Rat1 cells with P210/CDC24, P210/Luci, or P210/LacZ behaved in a similar fashion to mock-expressing cells. This is consistent with a recent report that claims that activation of Akt correlates with cell migration (35).

**Investigation of Mechanism**—

The DH sequence from Dbl appeared to substitute for the BCR DH sequence in the activation of P210BCR-ABL in terms of the ability to cause both cellular transformation and growth factor independence, but not in terms of the ability to cause survival against genotoxic agents. The DH domain could participate in the formation of a special molecular structure in P210BCR-ABL, independently or in concert with another portion of the molecule. This structure may play a role in binding to or recognizing signaling molecules such as substrate.

One possible mechanism would be that activated BCR-ABL is unable to autophosphorylate specific sites in the DH domain of BCR if it is replaced by other sequences. Although there are two tyrosine residues (BCR 561 and 598) in the DH region of FIG. 6. Tyrosine phosphorylation of STAT5, Shc, Vav1, and the p85 subunit of PI3K. Anti-STAT5 (A and B), anti-Shc (C and D), anti-Vav1 (E and F), and anti-p85 (G and H) immunoprecipitates from the same set of Ba/F3 cells shown in Fig. 5B were subjected to anti-STAT5 (B), anti-Shc (D), anti-Vav1 (F), anti-p85 (H), and anti-pTyr (A, C, E, and G) Western blotting.

FIG. 7. Antipoptotic activities of Ba/F3 cells expressing the DH mutants against serum starvation and cytotoxic agents. Ba/F3 cells shown in Fig. 5B were subjected to survival experiments against serum starvation (A) and cytotoxic reagents (B) including MMS, cytosine arabinoside (AraC), and cisplatin (CIS), and the means of three independent experiments are shown.
BCR that are conserved with neither Dbl nor CDC24, tryptic peptide mapping analyses of in vitro autophosphorylated P210/WT, P210/Dbl, and P210/CDC24 revealed almost no difference (Fig. 9). Coexpression studies of P210BCR-ABL with a BCR mutant (23) lacking the SH2-binding domain alone (BCRΔ162–413) or lacking both the SH2-binding domain and the DH domain (BCRΔ162–631) showed that neither of the mutants can be tyrosine-phosphorylated (data not shown).

By utilizing the yeast two-hybrid system, we also examined the second possibility regarding whether or not there is an interaction between the ABL SH2 domain and the DH domain because poor activation of the PI3K/Akt pathway has also been reported in the SH2 domain mutant (13). Under conditions in which the SH2 domain binds to the full-length P210BCR-ABL and the DH domain binds to XPB (20), we could observe no interaction between the SH2 domain and the DH domain.

We have previously reported the DH domain-mediated binding of BCR, but not of Dbl or CDC24, to XPB, suggesting that there is a specific interaction of the DH domain with other molecules (21). With full-length P210BCR-ABL and P185BCR-ABL as bait, yeast two-hybrid differential screening was undertaken to search for molecules that interact with the DH domain of BCR in the context of P210BCR-ABL. Both tissue glutaminase and Arp2 were found to be positive in P210 bait but negative in P185. However, we could coimmunoprecipitate neither of them with P210 expressed in Rat1 cells or Ba/F3 cells in repeated experiments (data not shown). In addition, coexpression of tissue glutaminase and P210BCR-ABL did not change the autophosphorylation activity in vivo. We could observe no difference between P210/WT and P210/CDC24 (or P210/Dbl) in the amount of coimmunoprecipitated actin (data not shown).

**DISCUSSION**

Abrogation of biochemical and biological functions by replacement of the DH domain of BCR in P210BCR-ABL with that of CDC24 or with irrelevant sequences such as LacZ or luciferase was surprising because a naturally occurring physical deletion of the DH domain in P210BCR-ABL is found in P185BCR-ABL, an alternative form of BCR-ABL that is more transforming than P210 (2). This seemingly contradictory evidence suggests that P185BCR-ABL and P210BCR-ABL are distinct from each other in terms of structure-based activation status and that the simple presence or absence of the DH domain sequence in BCR-ABL cannot explain the activation
levels. In the context of P210BCR-ABL, this region plays an important role in activation. At least two aspects should be noted: structural significance and functional significance.

The occurrence of the large deletion spanning the inserted sequence in the DH region in P210/Luci and P210/LacZ expressed in Ba/F3 cells strongly suggests that those molecules are structurally unstable and that P210BCR-ABL cannot maintain the structure of such irrelevant sequences (possibly at the level of mRNA) because reverse transcription-polymerase chain reaction analysis of Ba/F3 cells that survived against IL-3 deprivation revealed that clones carrying an in-frame deletion of the inserted sequence gave rise to growth. P210/ CDC24, which was stably expressed in those cells, could not exert full biochemical and biological activities. The dramatic reduction of tyrosine phosphorylation of BCR-ABL substrates such as Shc, Vav1, the p85 subunit of PI3K, Crkl, and Cbl correlated well with decreased potentials in antiapoptosis and migration in Ba/F3 cells expressing P210/CDC24.

Protein-protein interactions are often mediated by modifications such as phosphorylation. It has been shown that P210BCR-ABL has different phosphorylation sites from those in P185, as judged by peptide mapping analysis (36). The exchange activity of the Vav1 protein that contains the DH domain has been reported to be activated by tyrosine phosphorylation (37). However, the site of phosphorylation does not fall in the DH domain. Results from peptide mapping studies, yeast two-hybrid assays, and coexpression studies of P210BCR-ABL and BCR mutants of the DH domain suggest that the DH domain has been reported to be activated by tyrosine phosphorylation (37). However, we have not succeeded in yeast two-hybrid system.

Acknowledgments—We thank Dr. Richard Cerione (Cornell University) and Dr. Y. Novijs (Gunma Medical School) for helpful discussions.

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