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Suppression of Human Colorectal Carcinoma Cell Growth by Wild-Type p53

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Mutations of the p53 gene occur commonly in colorectal carcinomas and the wild-type p53 allele is often concomitantly deleted. These findings suggest that the wild-type gene may act as a suppressor of colorectal carcinoma cell growth. To test this hypothesis, wild-type or mutant human p53 genes were transfected into human colorectal carcinoma cell lines. Cells transfected with the wild-type gene formed colonies five- to tenfold less efficiently than those transfected with a mutant p53 gene. In those colonies that did form after wild-type gene transfection, the p53 sequences were found to be deleted or rearranged, or both, and no exogenous p53 messenger RNA expression was observed. In contrast, transfection with the wild-type gene had no apparent effect on the growth of epithelial cells derived from a benign colorectal tumor that had only wild-type p53 alleles. Immunocytochemical techniques demonstrated that carcinoma cells expressing the wild-type gene did not progress through the cell cycle, as evidenced by their failure to incorporate thymidine into DNA. These studies show that the wild-type gene can specifically suppress the growth of human colorectal carcinoma cells in vitro and that an in vivo–derived mutation resulting in a single conservative amino acid substitution in the p53 gene product abrogates this suppressive ability.

ONE COPY OF THE SHORT ARM OF chromosome 17, which harbors the p53 gene, is lost in many human tumors, including those of the colon and rectum (*1–3*). In the majority of human colon carcinomas with allelic deletions of chromosome 17p, the remaining p53 allele contains a missense mutation (*3, 4*). In addition to colorectal carcinomas, p53 gene mutations have also been found in conjunction with chromosome 17p allelic deletions in tumors of the brain, breast, lung, and bone (*4–6*). These studies are consistent with the hypothesis that the normal (wild-type) p53 gene product may function as a suppressor of neoplastic growth, and that mutation or deletion, or both, of the wild-type gene inactivates this suppression. This hypothesis has been supported by studies in rodent cells. For example, p53 alleles are often rearranged or mutated as a result of viral integration events in Friend virus–induced mouse erythroleukemias (*7*). Additionally, in transfection studies, the wild-type murine p53 gene has been shown to inhibit the transforming ability of mutant p53 genes in rat embryo fibroblasts (*8*). Other studies, however, have suggested that expression of the wild-type p53 gene product is necessary (not inhibitory) for cell growth (*9, 10*). Thus, the effect of wild-type and mutant p53 genes on cell growth may depend on the cell type examined. We now show that expression of the wild-type p53 gene in human colorectal carcinoma cells dramatically inhibits their growth. Moreover, a p53 gene mutant cloned from a human colorectal carcinoma was biologically inactive in this respect, as it was incapable of inhibiting such growth.

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Table 1. Colony formation after transfection with wild-type and mutant p53 expression vectors. For each experiment, one or two 75-cm^2 flasks were transfected (*13*), and the total colonies counted after 3 to 4 weeks of selection in geneticin (0.8 mg/ml). Exp., experiment.

Cell line	Exp.	No. of geneticin-resistant colonies formed	
		pC53-SCX3 (mutant)	pC53-SN3 (wild-type)
SW837	1	754	66
	2	817	62
SW480	1	449	79
	2	364	26
RKO	1	1858	190
	2	1825	166
VACO 235	1	18	16
	2	26	28

The colorectal carcinoma lines SW480 and SW837, which are representative of 75% of colon carcinomas, have each lost one copy of chromosome 17p (including the p53 gene), and the remaining p53 allele is mutated (*3, 4*). The SW837 line contains an arginine to tryptophan mutation at codon 248 (*4*). The SW480 line contains two point mutations, arginine to histidine at codon 273 and proline to serine at codon 309 (*4*). The substitutions at codon 248 and 273 are typical of those observed in human tumors, occurring within two of the four mutation "hot spots" (*4*). For the transfection studies, we constructed a vector, pCMV-Neo-Bam, engineered to contain two independent transcription units (*11*). The first unit comprised a cytomegalovirus (CMV) promoter/enhancer upstream of a site for insertion of the cDNA sequences to be expressed, and splice and polyadenylation sites to ensure appropriate processing. The second transcription unit included a herpes simplex virus (HSV) thymidine kinase promoter/enhancer upstream of the neomycin resistance gene, allowing for selection of transfected cells in geneticin (*11*). A wild-type p53 cDNA was inserted into pCMV-Neo-Bam to produce pC53-SN3. Similarly, a vector, pC53-SCX3, expressing a mutant cDNA from human colorectal tumor CX3, was also constructed. The only difference between pC53-SN3 and pC53-SCX3 was a single nucleotide (C to T) resulting in a substitution of alanine for valine at p53 codon 143 in pC53-SCX3 (*12*).

The constructs were transfected into SW837 and SW480 cells (*13*), and geneticin-resistant colonies were counted 3 weeks later. Cells transfected with pC53-SN3 formed five- to tenfold fewer colonies than those transfected with pC53-SCX3 in both recipient cell types (Table 1). In both

SW837 and SW480 cells, the number of colonies produced by the expression vector pCMV-Neo-Bam (without a p53 cDNA insert) was similar to that induced by the pC53-SCX3 construct.

These results suggested that the wild-type p53 gene inhibited the clonal growth of both the SW837 and SW480 cell lines; however, a significant number of colonies formed after transfection of the wild-type construct. If wild-type p53 expression were truly inhibitory to cell growth, one would expect that no colonies would form or that p53 expression in the colonies that did form would be reduced compared to that produced with the mutant p53 cDNA construct. To evaluate this issue, we expanded independent SW480 and SW837 colonies into lines, and ribonuclease (RNase) protection analysis was performed to determine the amount of p53 mRNA expressed from the exogenously introduced sequences. Twelve of 31 lines (38%) derived from transfection with the pC53-SCX3 construct were found to express the exogenous mutant p53 mRNA. This percentage was consistent with results expected in human cells transfected with a vector containing two independent transcription units (*14*). In contrast, no expression of exogenous p53 wild-type mRNA was seen in any of 21 clonal lines established from either SW480 or SW837 cells transfected with the pC53-SN3 vector (Fig. 1A). These RNase protection results were supported by analysis of the exogenous p53 DNA sequences within the clones. All of the p53-expressing clones derived from the pC53-SCX3 transfection contained an intact copy of the exogenous p53 gene (Fig. 1B). In contrast, in all the clones derived from the pC53-SN3 transfection, the exogenous p53 sequences were deleted or rearranged (Fig. 1B).

The results from individual clones were further supported by the analysis of pooled clones, in which numerous colonies could be simultaneously assessed. Forty or more clones from two to three separate transfection experiments were pooled and analyzed approximately 3 weeks after transfection. RNase protection studies showed substantial expression of exogenous mutant sequences in the pooled clones, whereas expression of wild-type sequences was not detectable (Fig. 2A). Results from Southern (DNA) blotting were consistent with the RNase protection studies, in that pooled colonies from the wild-type transfectants had no detectable unrearranged exogenous p53 sequences, in contrast to the intact p53 sequences in colonies derived from the mutant p53 cDNA expression vector (Fig. 2B).

The conclusions made from the above experiments are dependent on the assumption that p53 protein was produced in the transfected cell lines. Clones containing exogenous mutant p53 sequences produced p53 mRNA at a concentration 1.5 to 3.5 times higher than that produced by the endogenous p53 gene (Figs. 1A and 2A). Immunoblot analysis showed that there was a concomitant small increase in p53 protein expression in the transfectants (1.5- to 3-fold) compared to the untransfected cells (*15*). However, this increase was difficult to measure quantitatively, since these cells produced significant amounts of endogenous p53 protein that (unlike endogenous p53 mRNA) could not be distinguished from that produced by the vectors. To confirm that transfected human cells expressed p53 protein from our constructs, we studied an additional colorectal carcinoma cell line (RKO). Although RKO cells did not contain a mutation within the susceptible p53 coding sequences (*16*), they expressed low concentrations of p53 mRNA compared to normal colorectal mucosa or the other lines studied and did not produce detectable amounts of protein (*15*).

Results of colony formation assays in transfected RKO cells were similar to those in SW480 and SW837 cells. Colony formation by wild-type p53 gene transfectants occurred with a tenfold decrease in efficiency compared to the mutant p53 construct (Table 1). Immunocytochemical detection of p53 protein in transfected RKO cells (*17*) revealed that approximately equal numbers of cells expressed wild-type and mutant protein 6 hours after transfection. A twofold difference was found at 24 hours, and this

Table 2. Immunocytochemistry and [^{3}H]thymidine incorporation of transfected RKO cells. To determine p53 expression, we split RKO cells into eight flasks and individually transfected them with either pC53-SCX3 or pC53-SN3 (*13*). At the indicated times after transfection, cells from each flask were fixed and stained with a monoclonal antibody to p53 protein (*17*). At least 1500 cells were counted for each determination. To determine [^{3}H]thymidine incorporation, we split RKO cells into duplicate flasks and individually transfected them with either pC53-SCX3 or pC53-SN3 (*13*). Forty-six hours after transfection, the cells were incubated with [^{3}H]thymidine for 2 hours, then fixed and stained with a monoclonal antibody to p53 protein (*17*). Evaluation of thymidine incorporation in the transfected cells was performed as previously described (*19*). At least 50 p53-expressing cells and 400 p53-nonexpressing cells (determined immunocytochemically with antibody to p53) were assessed for each determination of [^{3}H]thymidine uptake.

Plasmid	Percent of cells expressing p53 protein at				Percent of cells incorporating [^{3}H]thymidine in	
	6 hours	24 hours	48 hours	96 hours	p53 expressors	p53 nonexpressors
pC53-SCX3	2.0	11	4.3	2.0	24	31
pC53-SN3	1.9	5.2	0.3	0.2	1.7	33

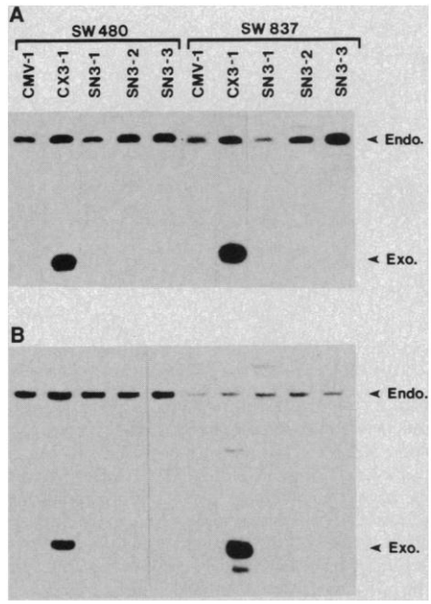

Fig. 1. (**A**) RNase protection analysis of transfected clonal lines. A labeled antisense p53 probe, which distinguishes between endogenous p53 mRNA and exogenous p53 mRNA, was hybridized with total cellular RNA from representative lines established from independent geneticin-resistant clones (*23*). After digestion with RNase A, the resulting hybridization products were separated by electrophoresis on denaturing polyacrylamide gels and autoradiographed (*24*). The labeled probe comprised nucleotides 1450 to 1788 relative to the p53 translation initiation site. Endogenous p53 mRNA included all of the p53 sequences represented in the labeled probe, so a 388-bp hybridization product (Endo.) was protected from RNase digestion. The exogenous p53 mRNA produced from the expression vector, however, only extended to nucleotide 1671; hybridization to exogenous p53 mRNA followed by RNase digestion therefore gave rise to a 221-bp fragment (Exo.). Clonal lines designated CMV, CX3, and SN3 were transfected with pCMV-Neo-Bam, pC53-SCX3, and pC53-SN3, respectively. (**B**) Southern blot analysis of transfected clonal lines. DNA from representative clonal lines (Fig. 1A) was digested with Bam HI, separated by electrophoresis on an agarose gel, transferred to nylon, and hybridized to a labeled p53 gene probe (*25*). The exogenous p53 gene from the expression vector was present in a 1.8-kb Bam HI fragment (Exo.) if it had not been rearranged in the cell. The endogenous p53 gene (Endo.) gave rise to a 7.8-kb Bam HI fragment.

difference increased with time (Table 2). These observations are consistent with the greater stability of mutant compared to wild-type p53 protein noted previously (*18*). However, transient mRNA expression was also significantly lower in the SN3 transfectants compared to the SCX3 transfectants at 48 and 96 hours (*15*), supporting the idea that RKO cells expressing wild-type p53 were at a selective disadvantage compared to those producing mutant p53 products.

To obtain additional evidence that cells expressing p53 were inhibited in their growth potential, we examined the effect of p53 gene expression on DNA synthesis in transfected RKO cells. Forty-eight hours after transfection, RKO cells were labeled with [^{3}H]thymidine for 2 hours. The cells were subsequently fixed, immunocytochemically stained for the presence of p53 protein, and autoradiographed (*19*). The number of cells undergoing DNA replication was only slightly lower in cells producing exogenous mutant p53 protein than in cells that did not express any detectable p53 protein. Expression of the wild-type protein, however, dramatically inhibited the incorporation of thymidine (Table 2).

These results all suggested that wild-type p53 exerted an inhibitory effect on the growth of carcinoma cells in vitro. To evaluate whether this inhibitory effect was cell type–specific, we transfected colorectal epithelial cells derived from a benign tumor of the colon (the VACO 235 adenoma cell line). Previous studies have shown that most adenomas contain two copies of chromosome 17p and express wild-type p53 mRNA at concentrations similar to that of normal colonic mucosa (*1, 15*). Analogously, the p53 alleles of the VACO 235 cell line were sequenced and found to be wild type (*16*), and the expression of p53 mRNA was found to be similar to that of normal colorectal mucosa (*15*). In contrast to the results seen with SW480, SW837, and RKO cells, the pC53-SN3 and pC53-SCX3 constructs produced similar numbers of geneticin-resistant colonies after transfection of the VACO 235 line (Table 1). We considered, however, that the most definitive test for differential growth inhibition by wild-type versus mutant p53 genes involved analysis of exogenous p53 expression in pooled transfectants. Through such analysis, a large number of colonies could be examined simultaneously and the expression of exogenous mutant and wild-type p53 genes directly compared. Striking differences in the relative expression from the transfected genes were seen in all three carcinoma cell lines tested. VACO 235 transfectants, however, expressed similar amounts of exogenous p53 mRNA from either pC53-SN3 (wild-type) or pC53-SCX3 (mutant) p53 constructs (Fig. 2A).

In summary, our results suggest that expression of the wild-type p53 gene in colorectal carcinoma cell lines was incompatible with proliferation. The inhibitory effects of wild-type p53 were specific in two ways. First, a single point mutation in a p53 gene construct abrogated its suppressive properties as measured by three separate assays (colony formation, exogenous p53 expression in transfected clones, and thymidine incorporation). The CX3 mutant provided a control for gene specificity, as it contained only one conservative mutation, resulting in a substitution of one hydrophobic amino acid (alanine) for another (valine) at a single codon. Second, the growth-suppressive effect of the wild-type p53 construct was cell type–specific. Introduction of the wild-type vector into the VACO 235 adenoma cell line had no measurable inhibitory effect compared to the mutant p53 vector. There are several differences between the cell lines that could account for the differential effect of the introduced vectors. Regardless of the basis for the difference, the results with the VACO 235 cell line minimize the possibility that the wild-type p53 construct had some nonspecific, toxic effect on recipient cells; the effect was cell type–dependent.

The transfection and expression results of Table 1 and Fig. 2A suggest that cells at the premalignant stages of tumor progression (VACO 235) may be less sensitive to the inhibitory effects of wild-type p53 than malignant cells (SW480, SW837, and RKO). This hypothesis is consistent with previous results that suggest the wild-type p53 is less inhibitory to the growth of normal rat embryo fibroblasts than to their oncogene-transfected derivatives (*8*). This sensitivity may only be relative: expression of the wild-type gene at high concentrations might inhibit the growth of any cell type, including non-neoplastic cells, by overwhelming normal regulatory processes such as phosphorylation (*20, 21*). Genetic alterations that occur during the progression of colorectal tumors (*22*) may increase the sensitivity of cells to p53 inhibition, making wild-type p53 expression a key, rate-limiting factor for further tumor growth and expansion. At this point, and not before, mutations in the p53 gene would confer a selective growth advantage to cells in vivo, which would explain the frequent occurrence of p53 gene mutations and allelic loss only in the more advanced stages of colorectal tumorigenesis (*1, 22*).

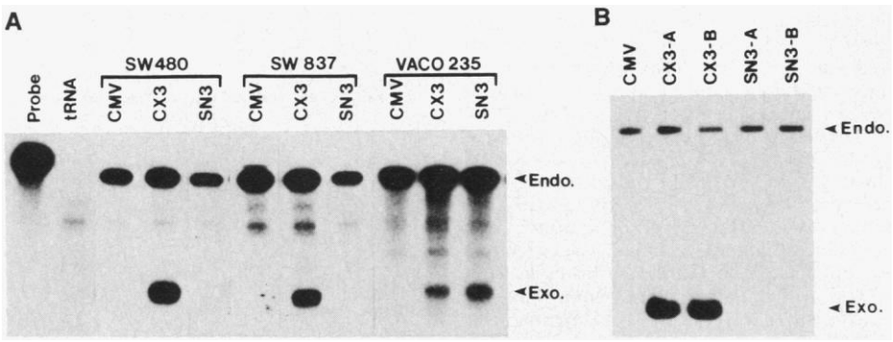

Fig. 2. (**A**) Expression analysis of pooled clones. One to four flasks containing a total of at least 40 independent geneticin-resistant clones transfected with pC53-SCX3 or pC53-SN3 (designated CX3 and SN3, respectively) were pooled for RNA preparation (*23*). Geneticin-resistant clones formed after transfection of pCMV-Neo-Bam, a vector devoid of p53 sequences (designated CMV), were used as a negative control. RNase protection was performed as described in Fig. 1A. Endogenous and exogenous p53 mRNA are designated as Endo. and Exo., respectively (see legend to Fig. 1A). (**B**) Southern blot analysis of SW480 pooled clones. DNA from pooled clones of SW480 cells was digested with Bam HI, separated by agarose gel electrophoresis, transferred to nylon, and hybridized with a labeled probe from the p53 gene as described (*25*). The lanes represent pooled clones from SW480 cells transfected with the following: lane CMV, pCMV-Neo-Bam; lanes CX3-A and CX3-B, pC53-SCX3 (two independent pools); and lanes SN3-A and SN3-B, pC53-SN3 (two independent pools). The 7.8-kb fragment from the endogenous p53 gene is indicated as Endo., and the 1.8-kb fragment from the exogenously introduced DNA is indicated as Exo.

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11. The expression vector pCMV-Neo-Bam was derived from plasmid BCMGNeo-mIL2 [H. Karasuyama, N. Tohyama, T. Tada, *J. Exp. Med.* **169**, 13 (1989)] by excision of the human beta globin sequences and bovine papilloma virus sequences with Bam HI and Not I. Next, the interleukin 2 (IL-2) sequences present at the unique Xho I site were removed, and the Xho I site was changed to a Bam HI site by linker addition. The vector included CMV promoter/enhancer sequences, which could drive expression of the insert at the Bam HI site, and splicing and polyadenylation sites derived from the rabbit beta globin gene, which ensured proper processing of the transcribed insert in the cells. A pBR322 origin of replication and beta-lactamase gene facilitated growth of the plasmid in *Escherichia coli*. The plasmid conferred geneticin resistance through expression of the neomycin resistance gene under separate control of an HSV thymidine kinase promoter.
12. A 1.8-kb Xba I fragment, extending from nucleotide −130 to 1671 relative to the translation initiation site, was isolated from wild-type or CX3 cDNA clones (*3*). The fragment was blunt-ended with the Klenow fragment of DNA polymerase, ligated to Bam HI linkers, and cloned into the unique Bam HI site in the expression vector pCMV-Neo-Bam.
13. SW480 and SW837 were obtained from American Type Culture Collection (ATCC). RKO cells were obtained through the generosity of M. Brattain. VACO 235 cells are described by J. K. V. Willson *et al.* [*Cancer Res.* **47**, 2704 (1987)]. For transfection, carcinoma cells at 30 to 60% confluence were incubated in a 75-cm^2 flask in 6 ml of Optimem (Gibco) with 5 μg of plasmid DNA and 30 μg of lipofectin [P. L. Felgner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7413 (1987)]. After 5 to 16 hours, the Optimem was replaced with Dulbecco's or McCoy's 5A medium containing 10% fetal calf serum. Selection in geneticin (0.8 mg/ml) began 36 to 48 hours after transfection for colony formation assays. Electroporation was used to transfect VACO 235 cells essentially as described by H. Potter, L. Wier, and P. Leder [*Proc. Natl. Acad. Sci. U.S.A.* **81**, 7161 (1984)].
14. Previous studies have shown that, in contrast to rodent cells, primate cells are able to integrate only a small amount of foreign DNA (approximately 6 kb), so that only 10 to 30% of clones selected for the expression of one transcription unit also contain the second unit in an intact form [F. Collabere-Garapin, M. Rhyiner, I. Stephany, P. Kourisky, A. Garapin, *Gene* **50**, 279 (1986); J. H. J. Hoeijmakers, H. Odijk, A. Westerveld, *Exp. Cell Res.* **169**, 111 (1987); L. Mayne *et al.*, *Gene* **66**, 65 (1988); S. W. Dean, L. Kincla, H. R. Sykes, A. R. Lehmann, I. A. Wise, *Exp. Cell Res.* **183**, 473 (1989)].
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16. The p53 gene sequences in exons 5, 6, 7, 8, and 9 were examined essentially as described in (*4*). All previously noted point mutations in p53 genes have involved one of these exons (see *3–5*).
17. Approximately 5 × 10^4 cells were cytocentrifuged onto polylysine-coated slides, fixed for 10 min in formalin, and permeabilized for 5 min in 0.5% Triton X-100. A mouse monoclonal antibody against human p53 protein (Ab1801) in combination with the ABC immunoperoxidase system (Vector Laboratories), was used for immunocytochemical detection of p53 protein [L. Banks, G. Matlashewski, L. Crawford, *Eur. J. Biochem.* **159**, 529 (1986)]. Ten to 20 randomly selected microscopic fields were analyzed per slide.
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19. Cells were grown for 2 hours in McCoy's 5A medium with 10% fetal calf serum and [^{3}H]thymidine (10 μCi/ml of 50 Ci/mmol, New England Nuclear). After immunocytochemical staining (*17*), slides were dehydrated in ethanol, dipped in NTB-2 emulsion (Kodak), and exposed for 2 weeks at 4°C. Autoradiographs were developed for 2 min in D-19 and stabilized in Rapid Fix (Kodak).
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23. Total cellular RNA was isolated by the acid guanidium extraction method [P. Chomczynski and N. Sacchi, *Ann. Biochem.* **162**, 156 (1987)].
24. RNA (15 μg) from each sample was used in RNase protection experiments. A ^{32}P-labeled RNA probe comprising nucleotides 1450 to 1788 relative to the p53 translation initiation site was generated in vitro from a p53 cDNA subclone in Bluescript with T7 polymerase. Ribonuclease protection was performed as previously described [E. Winter, F. Yamamoto, C. Almoguera, M. Perucho, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7575 (1985); R. M. Myers, Z. Larin, T. Maniatis, *Science* **230**, 1242 (1985)]. Autoradiographs were exposed for 16 to 20 hours.
25. DNA purification, restriction endonuclease digestion, electrophoresis, transfer, and hybridization were performed as described (*1, 3*). The hybridization probe was a 1.8-kb Xba I fragment of p53 cDNA (*12*).
26. We thank A. Preisinger, K. Molkentin, and J. Jackson for technical assistance. This work was supported by grants GM 07184, GM 07309, CA 43703, CA 45967, CA 43460, CA 51504, and CA 35494 from the NIH.

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A β_3 Integrin Mutation Abolishes Ligand Binding and Alters Divalent Cation–Dependent Conformation

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The ligand-binding function of integrin adhesion receptors depends on divalent cations. A mutant $\alpha_{IIb}\beta_3$ integrin (platelet gpIIb/IIIa) that lacks ligand recognition shows immunologic evidence of a perturbed interaction with divalent cations. This was found to be caused by a G → T mutation that resulted in an Asp^{119} → Tyr^{119} substitution in the β_3 subunit. This residue is proximal to bound ligand and is in a conserved region among integrins that are enriched in oxygenated residues. The spacing of these residues aligns with the calcium-binding residues in EF hand proteins, suggesting interaction with receptor-bound divalent cation as a mechanism of ligand binding common to all integrins.

CELL-CELL AND CELL-MATRIX ADHESIVE interactions are essential to development, inflammation, hemostasis, and immune recognition. The integrins are a broadly distributed family of structurally related receptors that contribute to these adhesive reactions by recognition of a multiplicity of extracellular matrix protein ligands including laminin, collagens, fibrinogen, and bone sialoprotein (*1*). In addition, integrins participate in cell-cell interactions by recognition of integral membrane protein ligands including the intercellular adhesion molecules ICAM-1 and ICAM-2 and vascular cell adhesion molecule–1 (VCAM-1) (*2*). Although the integrins differ in ligand recognition specificity, a requirement for millimolar concentrations of physiologic divalent cations is common to the primary recognition function of all integrins (*3*). This dependence of function on divalent cations can be attributed to a low-affinity divalent cation–binding site within the integrins, because millimolar Ca^{2+} or Mg^{2+} can modulate the conformation of a prototype integrin, platelet membrane glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$ also known as gpIIb/IIIa), which is detectable by a monoclonal antibody (MAb) PMI-1 (*4*). Loss of the epitope recognized by this MAb directly correlates with the capacity of $\alpha_{IIb}\beta_3$ to bind fibrinogen. The Cam variant of Glanzmann's thrombasthenia (*4*) is an autosomal recessive hereditary disorder of $\alpha_{IIb}\beta_3$ that is associated with the inability of this integrin to recognize macromolecular (*4*) or synthetic peptide (*5*) ligands. In addition, divalent cations do not regulate the expression of the PMI-1 epitope in Cam platelets (*4*). These characteristics indicate that the presumptive mutation in the Cam receptor leads to defects in binding of both divalent cations and primary ligands. To elucidate the structural basis of integrin function, we identified the point mutation in $\alpha_{IIb}\beta_3$ that causes the Cam variant of Glanzmann's thrombasthenia.

Total RNA was isolated from platelets of normal donors and two affected siblings with Cam variant. For initial sequencing, we

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