Masarykova Univerzita v Brně

Přírodovědecká fakulta

Molekulární biologie a genetika

Charakteristika vazebných vlastností proteinů se zaměřením na křížové struktury

Habilitační práce

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Poděkování

Mé díky patří kolegům z Biofyzikálního ústavu Akademie věd České republiky. Velké díky též mým učitelům a zejména profesoru Emilu Palečkovi, který byl vedoucím mé diplomové práce a zasvětil mě do vědecké práce.

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OBSAH

1. Úvod

Moderní metody molekulární biologie a sekvenační projekty přináší velké množství informací o genetickém základu biologických procesů. Z těchto výsledků je ovšem zřejmé, že pouze samotná znalost sekvence DNA nám nezodpoví veškeré otázky týkající se životních dějů v živém organismu. V poslední době se ukazuje, že epigenetické modifikace a lokální DNA struktury se významně podílejí i na základních biologických procesech a na udržování buněčné stability.

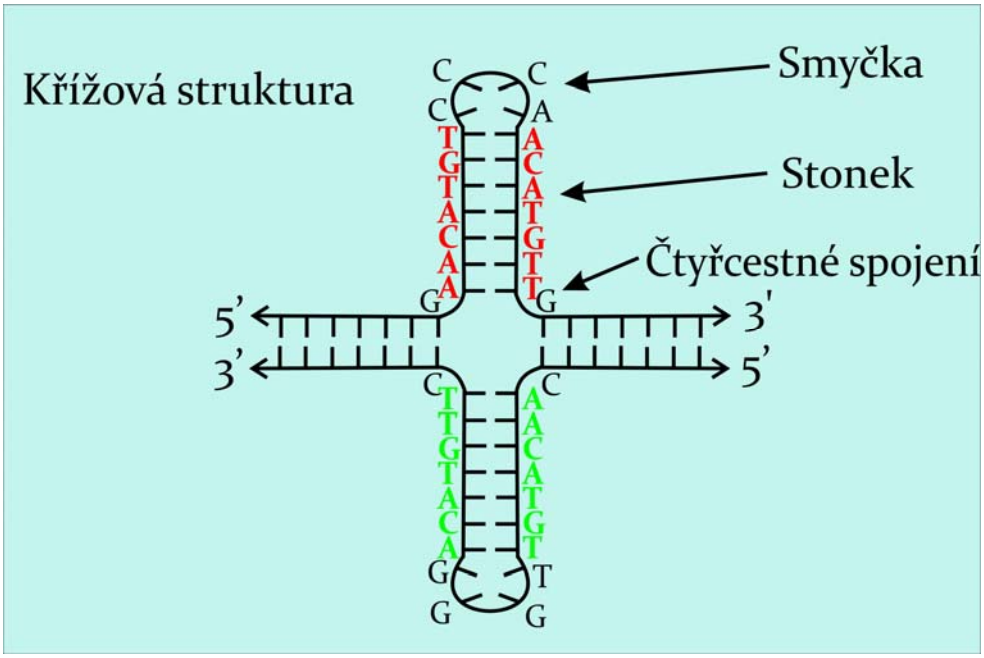
1.1. Lokální struktury DNA

Lokální struktury DNA byly popsány u všech živých organismů [1]. Topologie DNA hraje důležitou úlohu v mnoha biologických procesech jako regulace replikace, transkripce, rekombinace, kontroly genové exprese, organizace genomu atd. [2]. Negativní superhelicita DNA může stabilizovat a indukovat vznik různých konformačních změn DNA. Bylo ukázáno, že vazba celé řady proteinů na DNA je závislá na nadšroubovicovém vinutí molekuly DNA. Lokální struktury DNA jsou také velmi často přímo specificky rozeznávány různými proteiny [3]. V závislosti na superhelicitě, sekvenci DNA a interakci proteinů s DNA mohou vznikat v molekulách DNA různé lokální struktury, jako jsou například křížové struktury, levotočivá DNA, triplexy a kvadruplexy [2, 4, 5].

1.1.1. Křížové struktury

Vznik křížových struktur je závislý na sekvenci nukleotidů a pro vytvoření křížové struktury je nutná přítomnost úplné či částečné inverzní repetice šesti nebo více nukleotidů [6, 7]. Inverzní repetice se vyskytují nenáhodně v DNA všech živých organismů, zejména v promotorových oblastech, v místech iniciace replikace aj. Křížové struktury mohou ovlivňovat stupeň superhelicity DNA, tvorbu nukleozómů a vznik dalších lokálních struktur v DNA [8, 9]. Křížové struktury mají několik strukturních elementů, které mohou být cílem pro interakci s proteiny. Bylo ukázáno, že mnoho proteinů se váže ke křížovým strukturám a rozpoznává překřížení DNA, čtyřcestné spojení DNA řetezců či jednořetězovou smyčku a ohyby DNA (obrázek 1). Strukturní změny v chromatinové DNA se objevují zároveň při procesech, které vyžadují uvolnění řetězců z dvoušroubovicové struktury, včetně procesů

replikace a transkripce DNA [8]. Při těchto procesech dochází k relaxaci napětí molekul DNA za vzniku alternativních DNA struktur [10, 11]. Superhelicita DNA má velký vliv na genovou expresi. U *E. coli* bylo ukázáno, že exprese genů může být rychle a významně ovlivněna superhelicitou [12]. Křížové struktury jsou důležité regulátory biologických procesů [2, 7]. Kromě nadšroubovicového vinutí je formování křížové struktury ovlivněno také teplotou, délkou repetic a složením bází v repeticích, zejména v místech počátečního přerušení vodíkových vazeb [13]. Sekvence vhodné pro tvorbu křížových struktur se vyskytují často poblíž počátků replikace, promotorů a dalších regulačních míst, což naznačuje význam tvorby křížových struktur na regulaci replikace a transkripce [8, 14].



Obrázek 1: Schéma křížové struktury ze sekvence z promotoru genu p21. Inverzní repetice v sekvenci je zvýrazněna barevně, upraveno dle [3].

Tvorba křížových struktur *in vivo* byla ukázána jak u prokaryot, tak u eukaryot, několika metodikami. Jako první byla křížová struktura popsána u plazmidů, kde negativní superhelicita stabilizuje tvorbu křížové struktury. Plazmidy s negativním nadšroubovicovým vinutím obvykle obsahují křížovou strukturu *in vitro* i *in vivo* [15]. Delece sekvence tvořící křížové struktury v místě *ori* vede k redukci nebo nemožnosti replikace [16]. Podobně delece domény pro vazbu na křížovou strukturu proteinu 14-3-3 vede ke snížení vazby na počátky replikace a má vliv na iniciaci replikace u kvasinek [17]. K izolaci míst obsahujících křížové struktury byly úspěšně použity monoklonální protilátky proti křížovým strukturám [18].

Stabilizace křížových struktur pomocí monoklonálních protilátek 2D3 a 4B4 se specifitou k těmto strukturám vedla ke dvou až šestinásobnému zvýšení replikace *in vivo* [19]. Bylo také ukázáno, že protein 14-3-3σ je asociován s počátky replikace a podobně jako analog tohoto proteinu *S.cerevisiae* se váže na křížové struktury v DNA [20, 21]. Pomocí cílené mutační analýzy bylo demonstrováno, že vytvoření křížové struktury je potřebné pro regulaci genové exprese [22], a vznik křížových struktur v promotorové oblasti koreluje se zvýšením transkripce u určitých genů [23]. Hypo-metylace inverzních repetic ukazuje, že křížové struktury jsou hůře přístupné k metylázám [24]. Tyto výsledky dokazují důležitost křížových struktur a na jejich významný podíl při regulaci buněčných procesů.

1.1.2. Triplexy

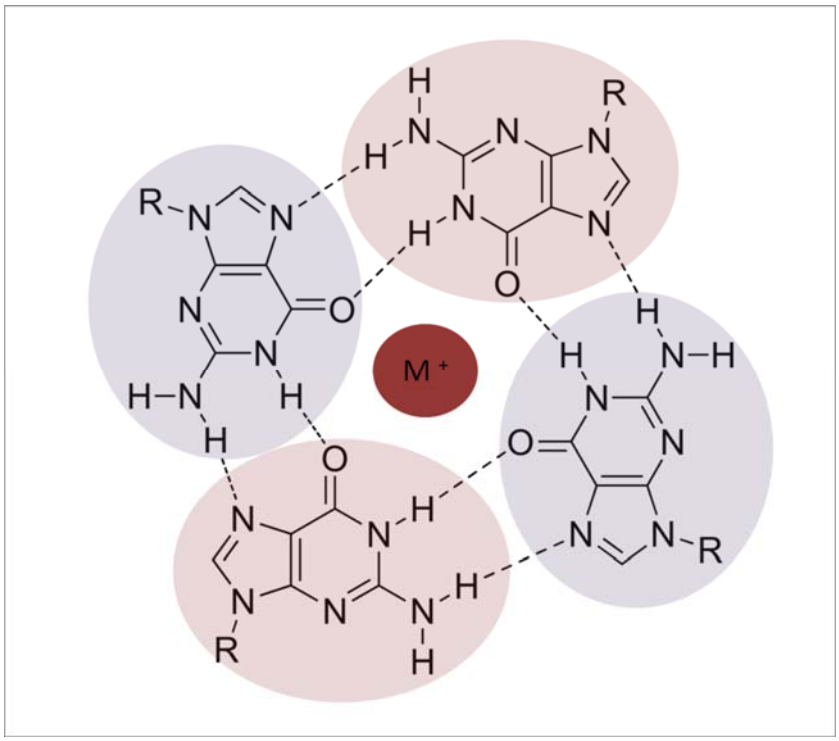
Triplexy, jak už název naznačuje, jsou tvořeny ze tří řetězců nukleových kyselin a obsahují sekvenční bloky homopyrimidinů nebo homopurinů. Báze v triplexové DNA jsou jednak spárovány Watson-Crickovým párováním, ale navíc i Hoogstenovým párováním [25]. Dle typu bází, které triplex zahrnuje, můžeme triplexy rozdělit na Y·R·Y (kde Y je pyrimidin a R je purin) a Y·R·R [25]. Triplexy typu Y·R·Y jsou obvykle tvořeny triádami bází T·A·T a C·G·C. U triplexů tohoto typu je nutná protonace cytozinu a vznikají pouze při nižším pH, označují se také jako H-DNA [26]. Dle počtu molekul, které tvoří triplex, můžeme triplexy rozdělit na intermolekulární (vznikají mezi různými molekulami DNA) a intramolekulární triplexy (vytváří se v rámci jediné molekuly DNA). U intramolekulárních triplexů dochází při jejich tvorbě ke vnoření jednoho z řetězců otočeného o 180 ° do velkého žlábku. Výsledná struktura tak obsahuje jednak triplexovou DNA, ale i nespárovaný čtvrtý řetězec, a dále také krátký jednořetězový úsek třetího řetězce mezi triplexem a duplexem [27]. Triplexové motivy se vyskytují častěji u eukaryot než u prokaryot a jsou lokalizovány například v intronech, promotorech a 5‘ a 3‘ nepřekládaných oblastech celé řady důležitých genů [28]. V lidských buněčných liniích byla přítomnost triplexů detekována pomocí monoklonálních protilátek [29, 30].

Obrázek 2: Schéma triplexové struktury, upraveno dle [31].

Triplexová DNA může blokovat některé základní biologické procesy, například H--DNA struktura *in vitro* efektivně blokuje *Taq* DNA polymerázu [32]. Nicméně bylo také ukázáno, že H-DNA způsobuje *in vivo* zastavení replikace a zvýšení tvorby jednořetězcových zlomů [33].

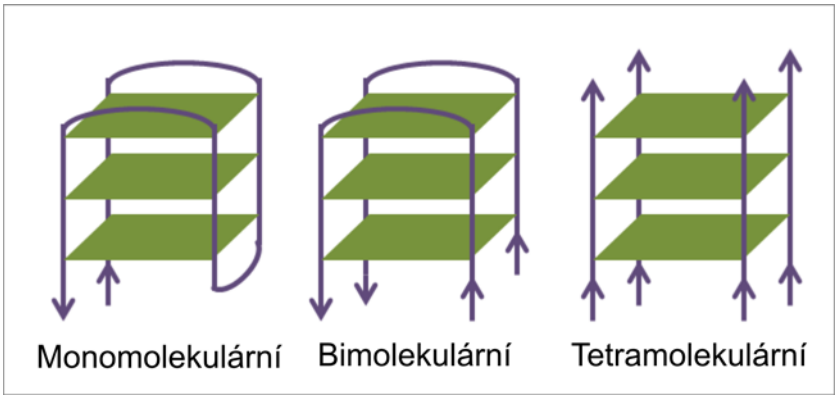
1.1.3. Kvadruplexy

V poslední době je intenzivně zkoumána také kvadruplexová DNA. Kvadruplexy mohou vznikat jak z DNA, tak z RNA molekul, zejména v oblastech bohatých na guanin (G--kvadruplexy, obrázek 3) a cytozin (i-motivy), nicméně tvorby kvadruplexů se mohou účastnit i ostatní nukleotidy. Nejčastější a nejlépe prozkoumanou skupinou kvadruplexů jsou ovšem G-kvadruplexy, které jsou typické například i pro lidské telomerní sekvence. Negativní náboj v centru G-kvadruplexu je neutralizován obvykle sodíkovým či draslíkovým iontem (obrázek 3) [34] a stabilita a uspořádání kvadruplexu jsou závislé na celé řadě faktorů [35].



Obrázek 3: Schéma Hoogstenova párování v G-kvadruplexu. Tetráda guanin (zvýrazněno barevně) je stabilizována iontem kovu (M+), upraveno dle [36].

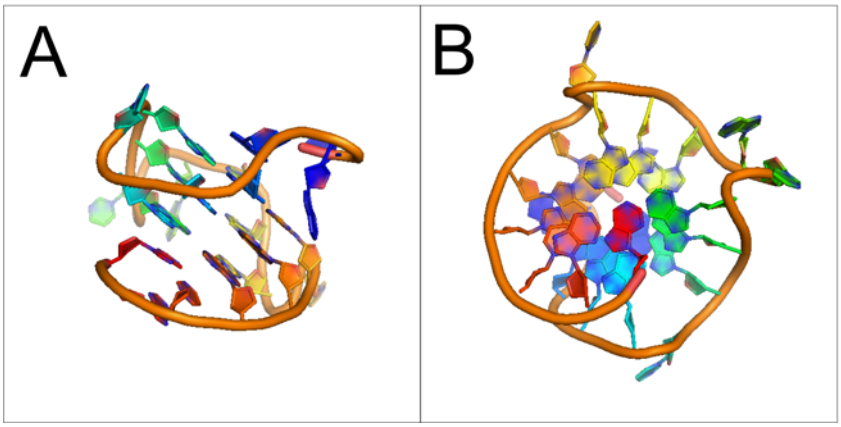
Dle počtu molekul, které kvadruplex tvoří, je můžeme rozdělit na intramolekulární a intermolekulární, a mohou být tvořeny jak jednou molekulou NA, tak dvěma či čtyřmi molekulami NA (obrázek 4) [37]. Dle orientace zúčastněných řetězců se G-kvadruplexy dále rozdělují na paralelní a antiparalelní. Zatímco RNA kvadruplexy se tvoří preferenčně v paralelní konformaci, DNA kvadruplexy mohou mít jak paralelní tak antiparalelní uspořádání, a v závislosti na experimentálních podmínkách mohou přecházet z jedné konformace do druhé. Velká variabilita G-kvadruplexů je ovlivňována: koncentrací solí, umístěním a délkou smyčky, která ke kvadruplexové struktuře přiléhá, přítomností nukleotidů a také počtem tetrád, které kvadruplex tvoří.



Obrázek 4: Schéma tvorby kvadruplexů z různého počtu řetězců DNA, zeleně jsou znázorněny tetrády guaninů, šedá reprezentuje cukr-fosfátovou kostru, upraveno dle [36].

Asociace guaninu byla pozorována už na konci 19. století, nicméně krystalografická studie ukázala uspořádání do G-kvartetu poprvé v roce 1962 [38]. Jednou z nejdříve objevených a charakterizovaných kvadruplexových sekvencí byla lidská telomerová sekvence [39]. Nicméně posléze byla popsána přítomnost oblastí bohatých na guanin s možností tvorby kvadruplexů v celé řadě sekvencí, včetně v promotorech některých onkogenů, a možnost tvorby G-kvadruplexů byla prokázána *in vitro*. Sekvenační data v současnosti umožňují analýzu celých genomů na přítomnost sekvencí s potenciálem tvorby kvadruplexů. Existuje několik programů pro tuto predikci [40–42]. Pomocí těchto algoritmů bylo ukázáno, že v lidském genomu je přibližně 376 000 sekvencí s potenciálem tvorby G-kvadruplexu [43]. Kromě telomerových oblastí jsou tyto sekvence často lokalizovány v promotorových oblastech onkogenů.

Struktura mnoha potenciálních kvadruplexů byla charakterizována pomocí NMR, X-ray (obrázek 4) a CD spektroskopie. Tyto metodiky potvrdily možnost oligonukleotidových řetězců tvořit kvadruplexové struktury *in vitro*.



Obrázek 5: Struktura G-kvadruplexu v místě NHE III regionu lidského c-Myc promotoru (PDVid: 1XAV), A – pohled zboku, B – pohled zespodu. Oranžová představuje cukr-fosfátovou kostru, guaninové báze formující tetrády jsou lokalizovány ve středu, upraveno dle [36].

Pro důkaz existence kvadruplexů *in vivo* se v současnosti používají dva základní přístupy. Díky specifické struktuře kvadruplexu byly charakterizovány ligandy, které jsou schopny selektivní vazby ke kvadruplexové struktuře [44]. Například molekula TMPyP4 (tetra-(N-methyl-4-pyridyl)porphyrin) byla popsána jako molekula, která se váže specificky na telomerní kvadruplexy a reguluje telomerázovou aktivitu [45], dále také snižuje expresi *c--Myc* genu a demonstruje tak přítomnost kvadruplexu v promotorové oblasti *c-Myc* genu [46]. Dalším příkladem je fluorescenční marker BMVC [3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide], který se také váže specificky ke kvadruplexové DNA se specifickým emisním maximem při vazbě na kvadruplex (575 nm) oproti fluorescenci při interakci s dvouřetězcovou DNA (545 nm). Pomocí fluorescenční mikroskopie byla lokalizována BMVC fluorescence v oblasti telomer [47]. Další důkazy přítomnosti kvadruplexové DNA *in vivo* využívají specifické protilátky [48–50], první protilátka se specifitou ke kvadruplexové DNA hf2 má dle testů nejméně 100× větší afinitu ke kvadruplexové DNA oproti dvouřetězcové DNA [48]. BG4 je další z protilátek se specifitou ke kvadruplexům, která byla charakterizována pomocí techniky ELISA. Rozpoznává jak DNA, tak RNA kvadruplexy, pomocí fluorescenční mikroskopie byla v buňkách lokalizována zejména v telomerických oblastech a zvýšené množství bylo detekováno také v jádře v průběhu replikace [51].

1.1.4. Levotočivá DNA

Oproti kanonické B-DNA, Z-DNA je levotočivá forma dvoušroubovice, ve které jsou báze téměř kolmo vzhledem k cukrfosfátové kostře, kde se střídá syn a anti konformace (zigzag pattern). Z toho důvodu je tvorba Z-DNA omezena na sekvence, ve kterých se střídají purinové a pyrimidinové sekvence, nejčastější vhodné sekvence opakování CG a AC bází. Z--DNA je nestabilní za standardních fyziologických podmínek a její tvorba *in vitro* vyžaduje vysoké koncentrace iontů, poprvé byla struktura demonstrována v roztoku 4M NaCl [52]. Tvorbu Z-DNA mohou zprostředkovat vysoká iontová síla, negativní nadšroubovicové vinutí, vazba proteinů, chemické modifikace a vazba lidandů [53]. Bylo ukázáno, že negativní superhelicita umožňuje tvorbu Z-DNA u prokaryot [54, 55], dalšími možnostmi stabilizace Z--DNA jsou také metylace DNA či přítomnosti sperminu a spermidinu. V současné době je studována konverze z B-DNA do Z-DNA pomocí kombinace metodiky FRET a magnetické pinzety. Bylo ukázáno, že i v případě malého superhelikálního napětí je možná tvorba Z-DNA v sekvencích CG *in vivo* [56]. Podobně inkorporace chemicky modifikovaných bází včetně brominace či metylace guaninu stabilizuje tvorbu Z-DNA a tato Z-DNA se tvoří i ve fyziologických podmínkách 150 mM NaCl [57].

Už v roce 1993 byla vyvinuta metodika k identifikaci Z-DNA vazebných proteinů [58]. Pomocí této metodiky byly izolovány proteiny s preferenční vazbou k této struktuře, patří mezi ně například enzym ADAR1, který je exprimován v centrálním nervovém systému [59]. Také byla demonstrována preferenční vazba k Z-DNA u proteinu E3L, který se nachází u poxvirů, včetně viru neštovic [60]. Mimo to bylo popsáno, že amyloidní protein, který je nadměrně exprimovaný při Alzheimerově chorobě, převádí Z-DNA zpět do B-DNA [61]. Dalším častým místem vazby proteinů mohou být místa tranzice B-DNA do Z-DNA. Analýza úplného lidského genomu ukázala, že oblasti bohaté na CG repetici se vyskytují velmi často, a to přibližně každých 3 000 párů bází, a korelují také s promotorovými regiony onkogenů [62]. Proto byla studována souvislost mezi transkripcí a výskytem Z- DNA a bylo zjištěno, že výskyt Z-DNA reguluje expresi některých onkogenů, jako jsou například lidský *ADAM - 12* a *c-Myc*. Z-DNA konformace byla demonstrována při transkripci genu *c-Myc*, naproti tomu, když byla DNA v této oblasti v B-formě, byla transkripce genu *c-Myc* vypnuta. Tyto výsledky také ukazují, že existence Z-DNA je závislá na fyziologických aktivitách buňky a může docházet k rychlé změně konformace ve vhodných DNA sekvencích [63].

1.2. DNA vazebné proteiny

Interakce proteinů s DNA patří mezi naprosto základní projevy biologických funkcí, a i proto je život jako takový často označován jako nukleoproteinový. DNA tvoří genetickou paměť živých organismů, ale bez správných interakcí s proteiny by nebyly možné ani základní biologické procesy. Proteiny však zároveň vytváří základní kostru pro uspořádání DNA v buňkách, mají regulační a enzymatické funkce, které umožňují vlastní předání informace, která je zapsána v sekvenci DNA.

Z hlediska specifity vazby proteinů na DNA můžeme tyto proteiny rozdělit na proteiny s nespecifickou vazbou k DNA, proteiny které se váží na DNA sekvenčně specificky a proteiny, které se váží specificky na různé struktury v DNA. Celá řada proteinů se váže na DNA bez sekvenční či strukturní specifity, tyto proteiny například umožňují organizaci DNA do chromatinu a patří mezi ně zejména histony. Velký význam v interakcích proteinů s DNA mají i proteiny, které jsou schopny vázat se sekvenčně či strukturně specificky k přesně definovaným oblastem v DNA, a díky tomu regulují mnoho esenciálních biologických procesů, včetně ontogeneze, oprav DNA, procesů stárnutí aj. Velmi dobře je charakterizována celá řada sekvenčně specifických proteinů. Patří mezi ně zejména transkripční faktory. Velmi často obsahují specifické proteinové motivy, jako zinkový prst, leucinový zip, dva helixy spojené krátkou smyčkou aj. [64]. Metody genetické manipulace umožňují adaptaci DNA vazebných proteinů na specifické sekvence [65]. Velký význam mají ovšem i interakce proteinů s lokálními DNA strukturami popsanými výše. Ukazuje se, že celá řada proteinů se váže právě na tyto neobvyklé DNA struktury s velkou preferencí. Byla ukázána preference různých proteinů jak ke triplexové DNA, tak ke kvadruplexům, Z-DNA a křížovým strukturám. Právě interakce různých proteinů ke křížovým strukturám DNA byla intenzivně studována, a budeme se jí proto v dalším textu věnovat detailněji.

1.2.1. Vazba proteinů ke křížovým strukturám

Inverzní repetice, ze kterých vznikají křížové struktury, jsou typickým rysem velkého množství regulačních míst v přirozených molekulách DNA. Není tedy překvapivé, že byla charakterizována celá řada proteinů, které se k těmto regulačním sekvencím specificky váží. Rozpoznání křížové struktury v DNA se zdá být rozhodující nejen pro stabilitu genomu, ale také pro další biologické děje. Z hlediska hlavních funkcí proteinů, které se váží na křížové struktury DNA, je můžeme rozdělit na: (a) enzymy rozpoznávající a štěpící křížové struktury,

(b) transkripční faktory a proteiny účastnící se oprav DNA, (c) proteiny důležité při replikaci a (d) proteiny asociované s chromatinem.

Tabulka1: Proteiny interagující s křížovými strukturami DNA

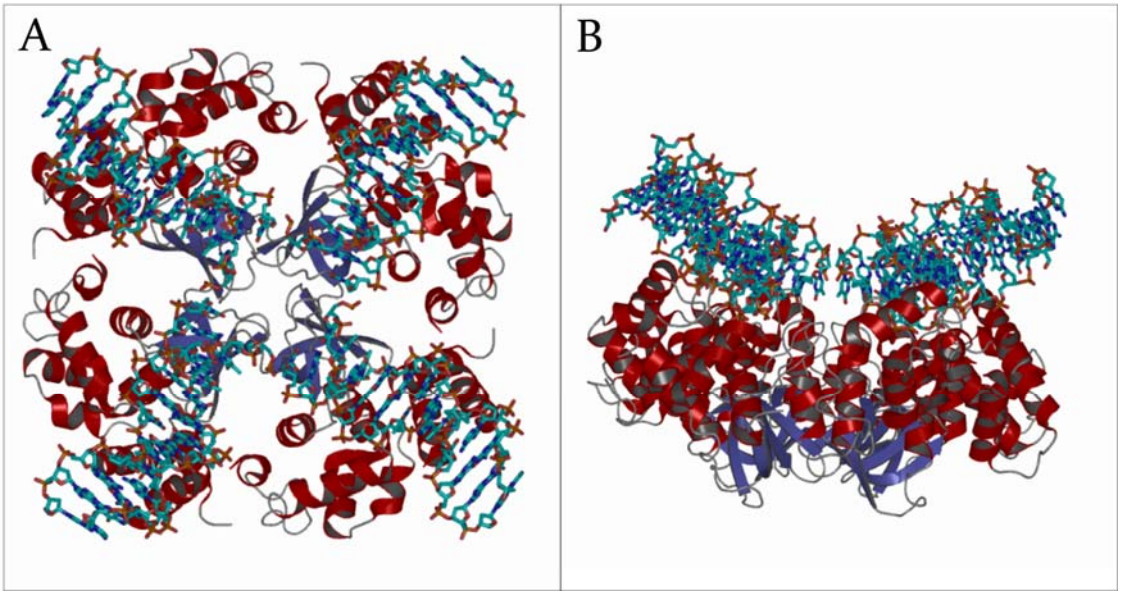
Protein	*Organismus*	*Reference*
Enzymy rozpoznávající a štěpící křížové struktury		
Rodina integráz		
RuvC	E.coli	[66–68]
Cce1	Kvasinky	[69]
Ydc2	S.pombe	[67]
A22	Coccinia virus	[70]
Integrázy		[71, 72]
Rodina restrikčních endonukleáz		
Endonukleáza I	Fág T7	[73–75]
RecU	G+ bakterie	[67, 76]
Hjc, Hje	Archea	[67, 77]
MutH		[78, 79]
Jiné		
Endonukleáza VII	Fág T4	[79, 80]
RusA	E.coli	[81]
Mus81-Eme1	Eukaryota	[82–85]
XPF, XPG proteiny		[86–88]
Transkripční faktory a proteiny účastnící se oprav DNA		
PARP-1	H. sapiens aj.	[89, 90]
BRCA1	H. sapiens aj.	[91–94]
P53	H. sapiens aj.	[95–101]
Bmh1	S.cerevisiae	[21]
14-3-3	H. sapiens	[20, 102]
HMG proteiny		[14, 103–105]
ER estrogen receptor		[106]
Proteiny asociované s chromatinem		
DEK	Savci	[107, 108]
BRCA1	H. sapiens aj.	[91–94]
HMG proteiny		[14, 103–105]

	Rad54		[109]
	Rad51ap		[110]
	Topoizomeráza I		[111, 112]
Replikace			
	S16	E.coli	[113]
	GF14	rostliny	[21]
	MLL	H. sapiens	[114, 115]
	WRN	H. sapiens	[116]
	14-3-3	H. sapiens	[20, 102]
	TRF2	H. sapiens	[117, 118]
	DEK	H. sapiens	[107, 108]
	DNA-PK	H. sapiens	[119]
	Rmi-1	H. sapiens	[120]
	Vlf-1	H. sapiens	[71]
	Crp-1	S. cerevisiae	[121]
	Helikázy 59, 44		[122]
	Hop1	S. cerevisiae	[123, 124]
	AF10		[125]

1.2.1.1. Enzymy rozpoznávající a štěpící křížové struktury

Přítomnost křížových struktur při replikaci může vést ke zlomům v DNA. Proteiny, které rozpoznávají a štepí křížové struktury, byly identifikovány u mnoha organismů od bakterií, přes archea, kvasinky až po savce [43]. Většina těchto enzymů může být rozdělena do dvou základních skupin [44]. Enzymy v první skupině se váží stejně na křížové struktury s různými sekvencemi, ale štepí pouze specifické cílové sekvence. Tato superrodina zahrnuje *E. coli* RuvC, kvasinkové integrázy, Cce1, Ydc2, and RnaseH proteiny.

Druhá skupina zahrnuje endonukleázy T7 RecU, enzymy Hjc a Hje, proteinovou rodinu MutH a příbuzné restrikční nukleázy. Tyto enzymy štepí DNA v místě čtyřcestného spojení nezávisle na sekvenci DNA. Komplex proteinu RuvA s křížovou strukturou byl charakterizován pomocí krystalografie (Obrázek 6) [79]. Tyto enzymy hrají důležitou úlohu při štepení cizorodé DNA a při udržování genomové stability. Funkce a specifita těchto proteinů byla publikována v několika přehledných článcích [79, 126–128].



Obrázek 6: Krystalová struktura terameru RuvA v komplexu s čtyřcestným spojením (PDBID 1C7Y) A – pohled shora, B – pohled zboku, upraveno dle [3].

1.2.1.2. Transkripční faktory a proteiny účastnící se oprav DNA

Udržování genomové stability je zprostředkováno několika nezávislými mechanismy. Promotorové oblasti genů jsou často charakterizovány přítomností obrácených repetic, které jsou schopné tvořit křížové struktury *in vivo*. Řada DNA-vazebných proteinů, například rodina HMGB-box [104], Rad54 [109], BRCA1 protein [91, 92], jakož i PARP-1 (poly (ADP-ribóza) polymeráza-1) [90] se váží relativně slabě na duplexovou DNA, ale váží se přednostně na křížové struktury. Kromě toho některé proteiny po vazbě na DNA mohou inicializovat vznik křížové struktury v místě vazby [90, 117]. Mezi proteiny, které se účastní oprav DNA a váží se ke křížovým strukturám, patří enzymy RuvA, RuvB [129, 130], helikázy DNA [122], protein XPG [88] a multifunkční proteiny jako proteiny HMG-box [131] BRCA1, 14-3-3 rodina proteinů, včetně jejich homologů Bmh1 a Bmh2 u *S. cerevisiae* a proteinu GF14 u rostlin.

PARP-1

PARP-1 je protein, který se vyskytuje významně v jádře buněk (cca jeden enzym na 50 nukleozómů) a obsahuje strukturu zinkových prstů. Kromě polyadenylace NA interaguje také s celou řadou proteinů. Má vysokou afinitu k poškozené DNA a stává se katalyticky

aktivní po vazbě na zlomy v DNA [132]. Při absenci poškození DNA vede přítomnost PARP--1 k rozrušení histonového komplexu s DNA a k vazbě regulačních faktorů [133]. Bylo zjištěno, že PARP-1 se může vázat na DNA vlásenky v heteroduplexu DNA. Atomová silová mikroskopie ukázala, že protein PARP-1 se přednostně váže do promotorové oblasti v superhelikální DNA, v níž se nacházejí symetrické sekvence vlásenky [134]. PARP-1 rozpoznává poškozenou DNA a váže se také na vícecestná spojení DNA [89]. PARP-1 se váže i na duplexovou DNA, ale kompetiční experimenty ukázaly, že nejlépe se váže na křížové struktury, dále na smyčky DNA a s nejmenší afinitou k duplexové DNA. Kromě toho bylo prokázáno, že vazba proteinu PARP-1 na DNA může měnit topologii DNA a podporovat tak ve vhodných sekvencích vznik křížové struktury [90].

P53

P53 je jedním z nejintenzivněji studovaných nádorových supresorových genů. A není to náhoda, mutace v genu *p53* je totiž nejčastější mutací u lidských nádorových onemocnění. Více než 50 % všech lidských nádorů obsahuje mutace p53 a inaktivace tohoto genu hraje kritickou roli v indukci maligní transformace [135]. Zásadní pro správnou funkci proteinu p53 je jeho vazba na cílové sekvence v promotorech celé řady genů. Tyto cílové sekvence p53 se skládají ze dvou kopií sekvence 5‘RRRC (A / T) (T / A) GYYY‘3. Konkrétní sekvence pro vazbu proteinu p53 může být tedy značně heterogenní, nicméně velmi často tyto sekvence tvoří obrácené repetice [136]. Experimenty *in vitro* ukázaly, že vazba proteinu p53 závisí na teplotě a délce fragmentu s cílovou sekvencí [137, 138]. Dále bylo však také ukázáno, že právě přítomnost inverzní repetice v cílové sekvenci p53 je důležitým determinantem vazby proteinu p53 k DNA, a to i *in vivo* [99, 139]. Známá a popsaná je též sekvenčně nespecifická vazba proteinu p53 na celou řadu DNA struktur, které nejsou v klasickém uspořádání B-DNA, jako jsou křížové struktury [140], ohnutá DNA [141], strukturně flexibilní chromatin DNA [142], hemicatenovaná DNA [143] nebo telomerní t-smyčky [144]. Cílové sekvence proteinu p53, které vytváří v superhelikální DNA křížovou strukturu, jsou preferenčním cílem proteinu p53 [100].

1.2.1.3. Proteiny asociované s chromatinem

Proteiny asociované s chromatinem pokrývají široké spektrum proteinů lokalizovaných v buněčném jádře. Tyto proteiny jsou důležité při modulaci chromatinu a jsou

často zapojeny i do procesů spojených s opravami DNA a replikací (DEK, BRCA1, HMG proteiny, Rad51, Rad51ap, topoizomerázy). Velmi důležitou skupinou jsou právě topoizomerázy, které se vyskytují u všech známých organismů a hrají zásadní roli v remodelaci topologie DNA. Tyto procesy jsou obzvláště důležité pro udržení stability genomu, protože ruší napětí molekuly DNA vznikající v průběhu transkripce a replikace. Bylo také ukázáno, že topoisomeráza I se váže na Hollidayovy spoje [145], topoisomeráza II rozpoznává a štěpí křížové struktury [146] a interaguje s proteinem HMGB1 [131]. Protein Rad54 hraje důležitou roli při homologní rekombinaci u eukaryot [147]. Kvasinkový a lidský protein Rad54 se specificky váží na Hollidayovy spoje a podporují migraci těchto překřížených molekul DNA [148]. Podobně proteiny RAD51AP1 a RAD51 mají afinitu k rozvětveným strukturám DNA [81]. Rozpoznávání rozvětvených struktur je důležitým krokem při homologní rekombinaci, které se tyto proteiny účastní [109].

DEK

Protein DEK patří mezi abundantní jaderné proteiny, které se vyskytují v jádře i ve více než milionu kopií [149]. Jeho interakce s transkripčními aktivátory a represory naznačuje, že protein DEK má úlohu při tvorbě transkripčních komplexů v promotorové a regulační oblasti [150]. Vazba DEK na DNA není sekvenčně specifická a protein se váže přednostně na superhelikální DNA a čtyřcestná spojení [108]. *In vitro* bylo ukázáno, že izolovaný protein DEK relaxuje pozitivní nadšroubovicové vinutí [107, 150]. Protein DEK má dvě vazebné domény DNA. První doména se nachází v centru proteinu a obsahuje evolučně konzervovanou část SAF (scaffold attachment factor). Druhá DNA-vazebná doména se nachází na C-konci, který může být posttranslačně modifikován fosforylací. Fosforylace proteinu DEK způsobuje snížení afinity proteinu k DNA a indukuje tvorbu multimerů proteinu [151, 152]. Samotná monomerní oblast SAF (zbytky 137–187) neinteraguje s DNA v roztoku, nicméně oblast aminokyselin 87–187 už je dostatečná pro vazbu na DNA s preferencí k čtyřcestným spojením DNA před lineární DNA. Tento fragment může vytvářet v přítomnosti DNA velké agregáty a je schopný vytvářet v uvolněné kruhové DNA negativní nadšroubovicové vinutí [153].

BRCA1

BRCA1 je multifunkční nádorový supresorový protein, který má roli v progresi buněčného cyklu, transkripci, opravách DNA a regulaci přestaveb chromatinu. Mutace v genu

BRCA1 je spojena s významným zvýšením rizika rakoviny prsu. Funkce BRCA1 pravděpodobně zahrnuje interakce s DNA a také s celou řadou proteinů. BRCA1 se často vyskutuje kolokalizovaný s proteinem RAD51 do diskrétních subjaderných ložisek, které se objevují při genotoxickém poškození DNA [154]. BRCA1 je také často kolokalizován s fosforylovaným H2AX v místech dvojitých zlomů DNA [155].

Centrální část lidského BRCA1 proteinu se silně váže na negativně nadšroubovicovou plazmidovou DNA s nativní superhelikální hustotou [91] a váže se s vysokou afinitou i na křížové struktury DNA [93]. BRCA1 také působí jako lešení pro asociované proteiny, zejména Rad51, který je odpovědný za homologní rekombinaci v somatických buňkách. Celý protein BRCA1 silně váže na nadšroubovicové plazmidové DNA a také na konce DNA [156]. Také fragment proteinu BRCA1 (230–534) se váže s vyšší afinitou ke křížové struktuře DNA ve srovnání s dvouřetězcovou a jednořetězcovou DNA [93]. Jako minimální oblast s DNA vazebnou afinitou byla definována oblast aminokyselinových zbytků 340–554 [94]. Ani 20násobný nadbytek lineární DNA nezpůsobil odvázání proteinu BRCA1 z místa křížové struktury [92]. Delece genu BRCA1 zabraňuje přežívání buněk po vystavení látek, které způsobují kovalentní vazbu mezi řetězci DNA v oblastech křížových struktur, jako je mitomycin C [157]. Tyto výsledky ukazují na důležitost rozpoznání křížových struktur proteinem BRCA1.

HMGB rodina

Proteiny HMG (high mobility group) se vyskytují ve velkém množství v eukaryotickém chromatinu. Skupina těchto proteinů zahrnuje tři hlavní rodiny: (a) proteiny HMGA (dříve HMG I/Y), které obsahují vazebné motivy A/T DNA, (b) proteiny HMGB (dříve HMG1/2), které obsahují doménu HMG-box, a (c) proteiny HMGN (dříve HMG14/17), obsahující doménu s afinitou k nukleozomu [158].

Bylo ukázáno, že HMGB se váží k DNA nezávisle na její sekvenci a mají preferenci pro různé lokální struktury DNA (čtyřcestné spojení, DNA minikroužky, cis-platinovanou DNA, atd.) oproti lineární DNA [159, 160]. U proteinu HMGB1 byla demonstrována velká afinita k DNA smyčkám [143]. Proteiny HMG jsou zapojeny do transkripce [111, 161, 162] a oprav DNA [131, 163, 164]. Bylo zjištěno, že protein T160 HMG je lokalizován v místech replikace [165]. Všechny HMG domény se váží preferenčně do čtyřcestných spojení DNA, a to v případě, že je tato struktura otevřená. Podmínky, které stabilizují uspořádanou konformaci X, významně oslabují vazbu HMG na DNA [166]. Tuto specifickou vazbu ruší

mutace Lys2 a Lys11 izolovaného boxu HMG, což ukazuje, že dané aminokyseliny jsou pro vazbu na DNA zásadní [167].

1.2.1.4. Proteiny podílející se na replikaci

Přítomnost vzniku přechodných křížových struktur v DNA koreluje s místy počátky replikace a transkripce [8]. Bylo prokázáno, že křížové struktury slouží jako rozpoznávací signály v místě počátku replikace DNA *in vivo* [102, 168, 169]. Existuje velké množství proteinů účastnících se replikace, které se váží na tyto struktury (viz tabulka 1). K nejlépe charakterizovaným proteinům v této oblasti patří proteiny 14-3-3, MLL (mixed-lineage, leukemia) a WNR (Werner syndrome ATP-dependent helicase).

Gen *MLL* kóduje transkripční faktor, který má homologní oblasti s HMG proteiny [114]. Chromozomální translokace11q23 se objevuje při akutních lymfoidních a myeloidních leukemiích a má za následek narušení tohoto genu a často také vytvoření chimérické fúze tohoto genu s různými jinými geny [115]. Jedna z domén tohoto proteinu se váže preferenčně ke křížovým strukturám v DNA a do AT bohatých oblastí genomu [114].

Protein WRN patří do rodiny RecQ evolučních konzervovaných 3 '→ 5' helikáz DNA [170]. Gen *WRN* kóduje protein o 162 kDa, který obsahuje 1 432 aminokyselin. Prokaryota a nižší eukaryota mají obvykle jeden člen helikáz RecQ, zatímco vyšší eukaryota mají více členů této rodiny, u člověka bylo identifikováno pět homologů. U proteinu WRN bylo prokázáno, že se váže na replikační vidlice a Hollidayovy spoje. [116].

Proteinová rodina 14-3-3 se skládá z vysoce konzervativní a široce distribuované skupiny dimerních proteinů, které se vyskytují u eukaryot v několika izoformách [171]. Existuje nejméně sedm různých genů *14-3-3* obratlovců, což vede k tvorbě devíti izoforem (α, β, γ, δ, ε, ζ, η, σ, τ), a nejméně dalších 20 bylo identifikováno u kvasinek, rostlin, obojživelníků a bezobratlých [102]. Pozoruhodným rysem proteinů 14-3-3 je jejich schopnost vázat velké množství funkčně odlišných signálních proteinů, včetně kináz, fosfatáz a transmembránových receptorů. S jejich pomocí proteiny 14-3-3 modulují celou řadu důležitých regulačních procesů, včetně mitogenní signální transdukce, apoptózy a regulace buněčného cyklu [172]. Proteiny 14-3-3 se nacházejí hlavně v jádře a jsou zapojeny do eukaryotické DNA replikace prostřednictvím vazby na křížové struktury v DNA [173]. Imunofluorescenční analýzy ukázaly přítomnost několika izoforem proteinu 14-3-3 vázaných na křížové struktury v buňkách HeLa [174]. Přímá interakce s křížovou DNA byla potvrzena

u 14-3-3 izoforem β, γ, σ, ε, a ζ [34]. 14-3-3 analogy se specifitou pro vazbu ke křížovým strukturám v DNA jsou také u kvasinek (Bmh1 a Bmh2) a rostlin (GF14) [21]. Kromě toho jsou proteiny 14-3-3 zapojeny do interakce s mnoha jinými transkripčními faktory.

1.3. Interakce nádorového supresoru p53 s DNA

Protein p53 je jedním z nejvýznamnějších nádorových supresorových proteinů. Byl objeven v roce 1979 a původně byl mylně považován za onkogen. Kotransfekce genu *Tp53* v roce 1989 totiž vedly k neoplastické transformaci buněk [175]. Později se ale ukázalo, že použité proteiny byly v mutované formě a že standardní varianta (wt) tohoto proteinu naopak takovéto transformaci brání [176], a byla odhalena jeho funkce nádorového supresoru. Systematické studie nádorových tkání odhalily, že protein p53 je nejčastěji mutovaný protein u lidských nádorových onemocnění. Toto zjištění vedlo k velmi intenzivnímu studiu tototo proteinu a celá řada jeho biochemických a funkčních vlastností byla detailně charakterizována. Díky své funkci je tento protein nazýván „Strážce genomu“ [177]. Svou úlohu má také při ontogenezi, ale za normálních okolností je jeho koncentrace v buňce nízká. V případě buněčného stresu či poškození DNA dochází k jaho stabilizaci a jeho koncetraci do jádra, v němž jako transkripční faktor reguluje expresi genů. Dle intenzity a typu poškození tak může docházet k zastavení buněčného cyklu nebo k p53-dependentní apoptóze [178]. Mutovaný protein p53 zpravidla neplní svoji funkci a dochází tak ke kumulaci chyb v genetické informaci, vedoucí často až v maligní přeměnu buňky. Jako transkripční faktor protein p53 zprostředovává svou funkci vazbou na DNA. Nejprve byla popsána sekvenčně specifická vazba na DNA, za niž je zodpovědná zejména centrální část proteinu, ve které se nachází i většina známých mutací proteinu p53 (obrázek 7) [179]. Nicméné pro jeho správnou funkci je nutná koordinace celého proteinu p53 a bylo ukázáno, že afinita celého proteinu p53 k DNA je řádově vyšší než pouze její centrální domény [180]. Kromě transkripční aktivace bylo ukázáno, že protein p53 se účastní i přímo reparačních procesů a rozeznává specificky celou řadu lokálních struktur DNA a poškození DNA. Zejména C-koncová oblast proteinu hraje v těchto procesech zásadní roli [181, 182]. Bylo ukázáno, že protein p53 rozeznává konce DNA, chybně párované řetězce, jednořetězcové mezery, ale také Hollidayovy spoje, křížové struktury, triplexovou a kvadruplexovou DNA [96]. Účastní se také homologní rekombinace a p53 fyzicky interaguje např. s proteinem Rad51, který je nezbytný pro správnost homologní rekombinace [183].

Obrázek 7: Domény proteinu p53, upraveno dle [184].

1.4. Choroby spojené s proteiny, které se váží na křížové struktury DNA

Rozpoznání křížových struktur v DNA je důležité pro stabilitu genomu a pro regulaci základních buněčných procesů. Disregulace ve štěpení čtyřcestných spojení může vést k translokacím DNA, delecím, chromozomové nestabilitě a karcinogenezi. Předpokládáme, že tvorba křížových struktur slouží jako marker pro správné načasování a zahájení některých velmi základních biologických procesů. Mutace a epigenetické změny, které mění možnosti vzniku křížové struktury, mohou mít na buněčné úrovni drastické následky. Není proto překvapivé, že dysregulace vazebných proteinů se specifitou vazby na křížové struktury je často spojena s patologickými stavy.

Celá řada proteinů, které mají preferenční vazbu ke křížovým strukturám jako proteiny p53, BRCA1, WRN a proto-onkogeny DEK, MLL a HMG, jsou velmi úzce spojeny se vznikem či progresí nádorového bujení. Některé z těchto proteinů hrají tak významnou roli, že jejich mutace a/nebo inaktivace způsobí závažnou genomovou nestabilitu. Například BRCA1 - / - myší embryonální kmenové buňky vykazují spontánní zlomy chromozomů a přecitlivělost na různá škodlivá činidla (například γ záření) a defekty spojené s nefunkčními opravami DNA. Mutace proteinu BRCA1 je také spojena s predispozicí ve vzniku malignit v prsní tkáni. Exprese dalšího nádorového supresoru proteinu p53 musí být přísně regulována. Vznik křížových struktur v cílových sekvencích pro protein p53 tak může být důležitou determinantou p53 transkripční aktivity.

HMG rodina proteinů obsahuje architektonické transkripční faktory, jejichž nadměrná exprese je vysoce korelována s karcinogenezí, zvýšenou malignitou a metastatickým potenciálem nádorů *in vivo* [95]. Proteiny 14-3-3 jsou spojeny s několika chorobami, včetně nádorových onemocnění, Alzheimerovy choroby, neurologických chorob, jako je Miller-

Diekerův syndrom a spinocerebelární ataxie typu 1, a spongiformní encefalopatie. Translokace genu DEK a vznik fúzního proteinu byla objevena u skupiny pacientů s akutní myeloidní leukémií a u vysokého procenta pacientů s autoimunitním onemocněním. Kromě toho hladina mRNA DEK je vyšší u transkripčně aktivních a proliferujících buněk a zvýšená hladina mRNA se často vyskytuje i u transformovaných nádorových buněk (6,7). Wernerův syndrom je autozomálně recesivní onemocnění charakterizované vlastnostmi předčasného stárnutí a vysokým výskytem neobvyklých nádorů a časným nástupem příznaků stárnutí [127].

Vazba proteinů na křížové struktury je důležitou součástí komplexní regulace na úrovni nukleových kyselin a hraje svou úlohu při základních buněčných procesech.

2. Výsledky

2.1. Vazba proteinu p53 k DNA

2.1.1. Regulace vazby proteinu p53 k DNA

Příloha 1: Pospisilova, S., Brazda, V., Kucharikova, K., Luciani, M.G., Hupp, T.R., Skladal, P., Palecek, E., and Vojtesek, B. (2004). Activation of the DNA-binding ability of latent p53 protein by protein kinase C is abolished by protein kinase CK2. Biochem J 378, 939-947.

Příloha 2: Brazda, V., Muller, P., Brozkova, K., and Vojtesek, B. (2006). Restoring wild-type conformation and DNA-binding activity of mutant p53 is insufficient for restoration of transcriptional activity. Biochem Biophys Res Commun 351, 499-506.

Příloha 3: Brazda, V., Jagelska, E.B., Fojta, M., and Palecek, E. (2006). Searching for target sequences by p53 protein is influenced by DNA length. Biochem Biophys Res Commun 341, 470-477.

Protein p53 je multifunkční protein, který zprostředkovává svou funkci nádorového supresoru především jako transkripční faktor. Patří mezi nejdůležitější regulátory buněčné proliferace, diferenciace a apoptózy. Protein p53 je také nejčastěji mutovaným genem u lidských malignit. Jako transkripční faktor se váže sekvenčně specificky k cílovým sekvencím DNA, typická cílová sekvence definovaná v roce 1992 se skládá ze dvou opakování 5‘PuPuPuC(A/T)(T/A)GPyPyPy-3‘ [185]. Tato sekvence se nachází v celé řadě promotorů a vazba proteinu p53 na tuto sekvenci umožňuje transaktivaci cílových genů. Mutace p53 zpravidla destabilizují protein a významně snižují jeho afinitu k DNA.

Protein p53 je strukturně flexibilní a může se nacházet v latentní konformaci, která se neváže na DNA, a v aktivní konformaci, která se váže na DNA sekvenčně specificky. Jedním z mechanismů regulace proteinu p53 je fosforylace specifických míst proteinu p53 [181]. S použitím gelové retardační analýzy jsme ukázali, že fosforylace proteinu p53 na jeho C-

-konci pomocí cdk2/cyklinuA na S315 a také fosforylace PKC na S378 efektivně stimuluje vazbu p53 na DNA. Naproti tomu fosforylace S392 pomocí CK2 má na vazebné vlastnosti proteinu mnohem menší efekt. Fosforylace pomocí CK2 kinázy dokonce inhibuje aktivaci proteinu p53 pomocí PKC kinázy. Tyto výsledky ukazují zásadní roli fosforylace proteinu p53 na jeho vazebné vlastnosti.

Dále jsme zkoumali různé mutanty proteinu p53 v lidských nádorových liniích – jejich konformaci, DNA vazebnou aktivitu a transaktivační aktivitu. Konformace jednotlivých mutantů byla analyzována pomocí protilátek rozeznávajících mutantní konformaci a konformaci wild-type. Zatímco například protein p53 s mutací R175H a R280K v linii SKBR3, respektive MDA-MB-231, vykazoval pouze aberantní konformaci, u mutantů proteinu p53 E285K a L194F z buněčných linií BT474 a T47D se lišilo množství proteinu rozeznávané konformačně specifickou protilátkou v závislosti na teplotě pěstování. U mutace E285K se při pěstování za 32 °C dokonce vyskytoval tento mutantní protein v konformaci rozeznávané výhradně protilátkou rozpoznávající nemutovanou p53. Podobně u experimentů s vazbou těchto proteinů na DNA záleželo velmi na teplotě, při které byly buňky kultivovány, a také na teplotě, při které se gelová retardační analýza prováděla. Při nízkých teplotách byla u některých mutantů zachována vazba k DNA, naproti tomu při 37 °C se mutanty vázaly k DNA jen minimálně. Detekce transaktivační aktivity *in vivo* ukázala, že i když některé mutanty jsou schopny vazby na DNA *in vitro* a mají částečně konformaci rozeznávanou protilátkou definovanou pro wt-p53, tak pouze mutant E285K v buněčné linii BT474 je schopen transaktivace při pěstování při 32 °C. Pomocí kompetičních experimentů jsme dále ukázali, že vazba proteinu p53 je výrazně ovlivňena délkou testované molekuly DNA. I když bylo místo sekvenčně specifické vazby k DNA lokalizováno do centrální části proteinu p53, C-terminální doména se váže také na DNA a je taktéž zodpovědná za vyhledávání cílového místa na dlouhých molekulách DNA.

2.1.2. Aktivace vazby protein p53 k DNA pomocí protilátek

Příloha 4: Pospisilova, S., Brazda, V., Amrichova, J., Kamermeierova, R., Palecek, E., and Vojtesek, B. (2000). Precise characterisation of monoclonal antibodies to the C-terminal region of p53 protein using the PEPSCAN ELISA technique and a new non-radioactive gel shift assay. J Immunol Methods 237, 51-64.

Příloha 5: Jagelska, E., Brazda, V., Pospisilova, S., Vojtesek, B., and Palecek, E. (2002). New ELISA technique for analysis of p53 protein/DNA binding properties. J Immunol Methods 267, 227-235.

Příloha 6: Brazda, V., Palecek, J., Pospisilova, S., Vojtesek, B., and Palecek, E. (2000). Specific modulation of p53 binding to consensus sequence within supercoiled DNA by monoclonal antibodies. Biochem Biophys Res Commun 267, 934-939.

Vazba proteinu p53 k jeho cílovým sekvencím je regulována celou řadou procesů, kromě postranslačních modifikací také vazbou jiných proteinů. K nejlépe prostudovaným interakcím patří zpětnovazebná inaktivace vazbou proteinu mdm2 k N-terminální části proteinu p53. Naproti tomu fosforylace C-terminální domény proteinu (viz. Kapitola 2.1.1), ale také interakce C-terminální domény s jinými proteiny vede často k aktivaci sekvenčně specifické vazby proteinu p53 k DNA. Z tohotu důvodu jsme podrobně charakterizovali sadu monoklonálních protilátek k C-terminální části proteinu p53. Pomocí testu PEPSCAN ELISA jsme definovali místa přesné interakce protilátek s C-terminální doménou. Tyto protilátky jsme využili dále pro imunohistochemické stanovení lokalizace proteinu p53 v buněčných liniích. Poté jsme charakterizovali vliv vazby těchto protilátek na vazbu proteinu p53 k DNA jednak pomocí radioaktivní gelové retardační analýzy, jednak pomocí neradioaktivního stanovení komplexů proteinů s DNA s fragmenty na agarózovém gelu. Ukázali jsme, že testované protilátky k C-koncové části proteinu aktivují protein p53 k sekvenčně specifické vazbě, na rozdíl od protilátky DO-1 rozeznávající N-konec proteinu p53. A také jsme demonstrovali, že nově testované protilátky rozeznávají oproti dříve používané protilátce 421 i protein p53, který je na konci fosforylovaný.

Sadu monoklonálních protilátek proti proteinu p53 jsme poté využili k nové ELISA technice stanovení DNA vazebných vlastností proteinu p53. Nejprve jsme navázali biotinem značené oligonukleotidy s p53 cílovou sekvencí na ELISA desku pokrytou streptavidinem. Poté jsme použili k promývání desky ELISA purifikovaný protein p53 nebo jaderné extrakty s p53 monoklonálními protilátkami. Po promytí a inkubaci se sekundární protilátkou konjugovanou s peroxidázou jsme detekovali intenzitu signálu a ABTS na readeru ELISA. Jako nejlepší pro studium vazebných vlastností DNA se ukázaly protilátky proti N-terminální části proteinu p53. Metodika je vhodná také pro kompetiční experimenty.

Jak bylo již demonstrováno, monoklonální protilátka 421 aktivuje protein p53 k vazbě na cílové sekvence [182]. Ukázali jsme, že i nově charakterizované protilátky proti C-konci proteinu jsou schopné aktivovat tuto vazbu na fragmenty DNA s cílovou sekvencí. Dále jsme ukázali, že protilátka Bp53-10 je schopna aktivovat vazbu proteinu p53 k cílové sekvenci i v superhelikální DNA. Naproti tomu protilátka DO-1 k N-koncové oblasti neaktivuje vazbu proteinu p53 na DNA. Naopak pokud byla přidána před C-terminální protilátkou, došlo dokonce k zablokování možnosti aktivace proteinu p53. Tyto výsledky ukazují, že regulace vazby proteinu p53 na DNA centrální částí proteinu je velmi komplexní proces regulovaný nejen aktivační C-koncovou doménou.

2.1.3. Vazba proteinu p53 k superhelikální DNA

Příloha 7: Palecek, E., Brazdova, M., Brazda, V., Palecek, J., Billova, S., Subramaniam, V., and Jovin, T.M. (2001). Binding of p53 and its core domain to supercoiled DNA. Eur J Biochem 268, 573-581.

Příloha 8: Fojta, M., Brazdova, M., Cernocka, H., Pecinka, P., Brazda, V., Palecek, J., Jagelska, E., Vojtesek, B., Pospisilova, S., Subramaniam, V., et al. (2000). Effects of oxidation agents and metal ions on binding of p53 to supercoiled DNA. J Biomol Struct Dyn, 177-183.

Příloha 9: Palecek, E., Brazda, V., Jagelska, E., Pecinka, P., Karlovska, L., and Brazdova, M. (2004). Enhancement of p53 sequence-specific binding by DNA supercoiling. Oncogene 23, 2119-2127.

Příloha 10: Pivonkova, H., Sebest, P., Pecinka, P., Ticha, O., Nemcova, K., Brazdova, M., Jagelska, E.B., Brazda, V., and Fojta, M. (2010). Selective binding of tumor suppressor p53 protein to topologically constrained DNA: Modulation by intercalative drugs. Biochem Biophys Res Commun 393, 894-899.

Kromě sekvenčně specifické vazby proteinu p53 na DNA bylo ukázáno, že se tento protein váže i na jednořetězcovou DNA a lokální struktury v DNA jako například na nespárované úseky, ohyby DNA [141], chromatinovou DNA [142], hemikatenovanou DNA [143], Hollidayovy spoje [96], tří- a čtyřcestná spojení [96] nebo telomerické smyčky [186].

Několik skupin také ukázalo, že protein p53 se váže na negativně a pozitivně nadšroubovicovou DNA [139, 187]. Předpokládalo se, že za preferenční vazbu na superhelikální DNA je zodpovědná C-terminální doména proteinu. Nicméně naše výsledky s centrální částí proteinu p53 ukázaly, že i tento protein je schopen vázat se preferenčně na nadšroubovicovou DNA. Preference oproti celému proteinu není tak výrazná, celý protein má cca 60× větší afinitu k superhelikální DNA oproti lineární DNA, zatímco centrální část proteinu má afinitu oproti lineární DNA pouze čtyřnásobnou. Vazba bývá nazývána jako strukturně selektivní vazba proteinu p53. Tato strukturně selektivní vazba je výrazně ovlivňována přítomností některých iontů a oxidací proteinu p53. I když v DNA vazebné doméně proteinu p53 je vázaný iont zinku mezi Cys176, Cys 238, Cys 242 a His 179 a jeho přítomnost je nutná pro efektivní vazbu proteinu na DNA, zvýšená koncentrace zinku v DNA vazebném pufru vede k výraznému snížení vazebné afinity proteinu p53 k superhelikální DNA. Ionty niklu a kobaltu naproti tomu snižují afinitu proteinu p53 k DNA až při vyšších koncentracích. Také oxidace proteinu p53, při které dochází k uvolnění iontu zinku z DNA vazebné domény, vede ke změně konformace proteinu p53 a výraznému snížení afinity proteinu p53 k DNA s cílovou sekvencí. Vazba na superhelikální DNA je iontovými podmínkami a oxidací ovlivněna méně, než vazba sekvenčně specifická.

Pomocí nové kompetiční analýzy komplexů p53 s DNA v agarózovém gelu jsme sledovali vliv superhelicity na vazbu proteinu p53 k cílovým místům v superhelikání DNA. Do plazmidu pBluescript jsme naklonovali cílová vazebná místa proteinu p53 z promotorů některých genů významně proteinem p53 regulovaných. Ukázalo se, že v kompetičním uspořádání protein p53 preferuje vazbu na cílová místa, která jsou v superhelikální DNA, a to výrazněji v případech, v nichž jsou tato cílová místa tvořena inverzními repeticemi a afinita oproti stejné sekvenci v lineárním stavu byla zvýšena až 3× (např. cílová sekvence z promotoru p21). Pokud p53 cílové sekvence nemají inverzní repetici, je preference pro superhelikální DNA jen malá (např. cílová sekvence z promotoru RGC). Dále jsme testovali vliv interkalačních činidel DNA na vazbu proteinu p53 k superhelikální DNA. V případě přidávání chloroquinu ke vzorku superhelikální DNA s negativním nadšroubovicovým vinutím dochází se zvyšující se koncentrací chloroquinu k postupné relaxaci DNA, s dalším zvyšováním koncentrace interkalátoru se poté relaxovaná DNA postupně opět zavinuje a vzniká pozitivní nadšroubovicové vinutí. Pomocí imunoprecipitace p53 komplexů DNA na magnetických kuličkách jsme sledovali preferenci ke kruhové molekule DNA s různým nadšroubovicovým vinutím. Ukázalo se, že při relaxaci DNA dochází k výraznému snížení

afinity pro kruhovou molekulu oproti lineárnímu fragmentu DNA, ale při převedení relaxované molekuly zvýšenou koncentrací interkalátoru do pozitivního nadšroubovicového vinutí dojde opět ke zvýšení preference pro protein p53. Jak negativní, tak pozitivní nadšroubovicové vinutí tedy může zlepšovat vazebnou afinitu proteinu p53 k DNA.

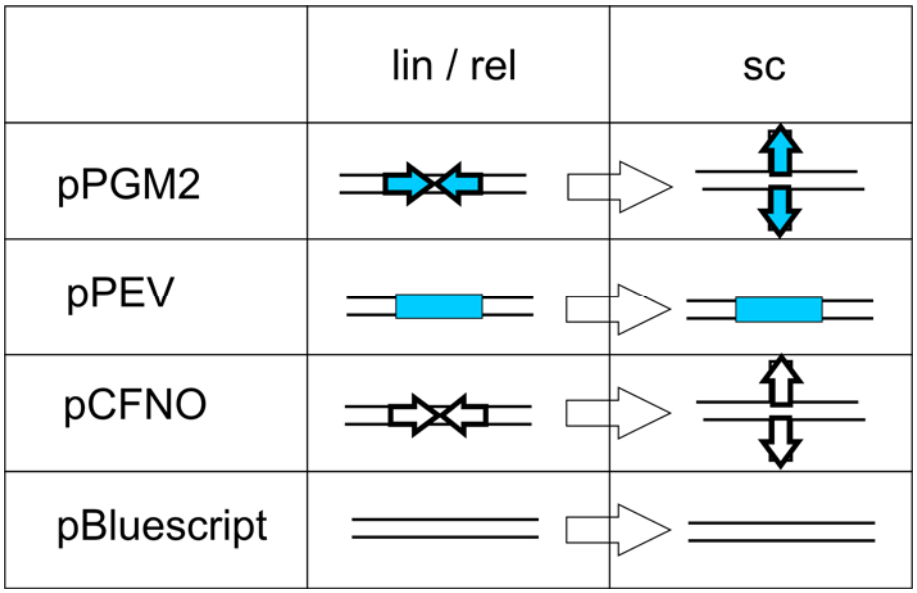
2.1.4. Vazba proteinu p53 k inverzním repeticím tvořícím křížové struktury

Příloha 11: Jagelska, E.B., Brazda, V., Pecinka, P., Palecek, E., and Fojta, M. (2008). DNA topology influences p53 sequence-specific DNA binding through structural transitions within the target sites. Biochem J 412, 57-63.

Příloha 12: Jagelska, E.B., Pivonkova, H., Fojta, M., and Brazda, V. (2010). The potential of the cruciform structure formation as an important factor influencing p53 sequence-specific binding to natural DNA targets. Biochem Biophys Res Commun 391, 1409-1414.

Příloha 13: Coufal, J., Jagelska, E.B., Liao, J.C., and Brazda, V. (2013). Preferential binding of p53 tumor suppressor to p21 promoter sites that contain inverted repeats capable of forming cruciform structure. Biochem Bioph Res Co 441, 83-88.

Při dalším výzkumu interakcí proteinu p53 s DNA jsme se zaměřili na přítomnost křížových struktur. Pro testování afinity proteinu p53 ke křížovým strukturám jsme připravili čtyři plazmidové konstrukty, které se lišily pouze v přítomnosti cílového místa pro protein p53 a v přítomnosti inverzní repetice s možností tvorby křížové struktury (obrázek 7). Měli jsme tedy k dispozici plazmid, který obsahoval cílovou sekvenci pro protein p53 s inverzní repeticí (pPGM2), plazmid, který obsahoval cílovou sekvenci bez inverzní repetice (pEV), plazmid, který obsahoval ideální inverzní repetici bez cílového místa pro protein p53 (pCFNO) a kontrolní plazmid bez těchto inzercí (pBluescript).



Obrázek 7: Schéma konstruktů použitých ve studii. Modrou barvou je označena cílová sekvence pro protein p53, šipky naznačují inverzní repetice, které vytváří v superhelikální DNA křížovou strukturu.

V nekompetičním uspořádání se protein p53 váže ke všem těmto plazmidům v superhelikálním stavu a pouze k fragmentům obsahujícím p53 cílovou sekvenci v lineárním stavu. V případě kompetičního experimentu má ovšem protein p53 nejvyšší afinitu k pPGM2, následuje pEV, pCFNO a nejslabší je vazba ke kontrolnímu plazmidu bez inzertů. Pomocí topoizomerázy jsme, kromě plazmidu s nativním nadšroubovicovým vinutím, připravili plazmidy s přesně definovaným nadšroubovicovým vinutím. Poté jsme provedli důkaz přítomnosti lokální struktury DNA v těchto topoizomerech pomocí nukleázy S1 a také pomocí dvourozměrné elektrororézy. Ukázalo se, že od superhelikálního vinutí $-\sigma=0,05$ vzniká v plazmidech lokální struktura DNA v místě inverzní repetice, která odpovídá přítomnosti křížové struktury v tomto místě plazmidu. Při kompetičním experimentu s topoizomery jsme pak mohli pozorovat výrazný skok v afinitě proteinu p53 k těmto plazmidům právě se zvyšující se superhelicitou od $-\sigma=0,05$. Tato tranzice nebyla pozorovatelná u plazmidů bez inverzní repetice. Z tohoto postulujeme, že k afinitě proteinu p53 přispívá významně právě vznik křížové struktury v p53 cílové sekvenci. Stejný efekt jsme poté pozorovali u dalších konstruktů, ve kterých jsme naklonovali do plazmidů p53 cílové sekvence z promotorů p53 dependentních genů. Se zvyšující se superhelicitou se protein p53 vázal lépe na cílová místa, zejména v případě možnosti tvorby křížové struktury v sekvenci. Detailněji jsme také zkoumali cílová místa proteinu p53 z promotoru genu p21. Pomocí

chromatinové imunoprecipitace jsme testovali, jak je protein p53 navázán na tento promotor po chemoterapeutickém poškození nádorových buněk. Protein p53 se tvořil významně zejména po poškození nádorové linie MCF-7 5-fluorouracylem, doxorubicinem a roscovitinem, což jsme detekovali pomocí Western Blotu a pomocí imunohistochemie, která nám ukázala po poškození buněk zřetelnou lokalizaci proteinu p53 v jádře buněk. V těchto buňkách docházelo poté také k výrazné expresi proteinu p21, zejména po použití 5--fluorouracilu a roscovitinu. Pomocí imunochromatinové precipitace jsme z těchto buněk vyizolovali p53-DNA komplexy. Nejvíce byl protein navázán právě na fragment z promotoru genu p21, který obsahuje nejen cílovou sekvenci, ale také inverzní repetice s potenciálem tvorby křížové struktury.

2.2. Preferenční vazba proteinu 14-3-3 k nadšroubovicové DNA

Příloha 14: Brazda, V., Cechova, J., Coufal, J., Rumpel, S., and Jagelska, E.B. (2012). Superhelical DNA as a preferential binding target of 14-3-3gamma protein. J Biomol Struct Dyn 30, 371-378.

Proteiny rodiny 14-3-3 patří mezi vysoce konzervovanou skupinu proteinů, která má u eukaryotních organismů několik izoforem. Proteiny 14-3-3 hrají klíčovou roli při replikaci a regulaci buněčného cyklu. Bylo ukázáno, že protein 14-3-3γ se váže silně na křížové struktury a je zásadní pro zahájení replikace. Testovali jsme vazebné vlastnosti proteinu 14-3-3γ k lineární a superhelikální DNA. Pomocí gelové retardační analýzy jsme ukázali, že tento protein se váže preferenčně k dlouhým molekulám DNA. Kompetiční experimenty poté ukázaly silnou preferenci pro superhelikální DNA. Pomocí konfokální mikroskopie jsme ukázali v buněčné linii HCT-116 kolokalizaci proteinů 14-3-3 s křížovými strukturami. Preference proteinu 14-3-3 k superhelikální DNA ukazuje na významnou roli proteinu 14-3-3 při vazbě na lokální struktury v DNA.

2.3. Vazebné vlastnosti nádorového supresoru proteinu BRCA1

Příloha 15: Brazda, V., Jagelska, E.B., Liao, J.C., and Arrowsmith, C.H. (2009). The Central Region of BRCA1 Binds Preferentially to Supercoiled DNA. J Biomol Struct Dyn 27, 97-104.

Protein BRCA1 je multifunkční nádorový supresor, který pomocí své interakce s dalšími proteiny a DNA má zásadní úlohu zejména při opravách DNA, transkripci a změnách struktury chromatinu. Provedli jsme detailní analýzu vazebných vlastností proteinu BRCA1 k linearní a superhelikální DNA. Centrální oblast proteinu BRCA1 se váže silně k negativně nadšoubovicové plazmidové DNA s nativní nadšroubovicovou hustotou. Už od nízkých koncentrací proteinu dochází k vazbě na superhelikální DNA a ke vzniku retardovaných komplexů, zatímco na lineární DNA se protein BRCA1 za těchto podmínek neváže. Komplexy proteinu BRCA1 se superhelikální DNA ještě více zpomalí přítomnost monoklonální protilátky proti proteinu BRCA1. V centrální oblasti jsou minimálně dvě nezávislé DNA vazebné domény, které se váží k superhelikální DNA.

2.4. Preferenční vazba proteinu IFI16 ke křížovým strukturám DNA

Příloha 16: Liao, J.C., Lam, R., Brazda, V., Duan, S., Ravichandran, M., Ma, J., Xiao, T., Tempel, W., Zuo, X., Wang, Y.X., et al. (2011). Interferon-inducible protein 16: insight into the interaction with tumor suppressor p53. Structure 19, 418-429.

Příloha 17: Brazda, V., Coufal, J., Liao, J.C., and Arrowsmith, C.H. (2012). Preferential binding of IFI16 protein to cruciform structure and superhelical DNA. Biochem Bioph Res Co 422, 716-720.

Jedním z proteinů, které mohou regulovat funkci proteinu p53, je protein IFI16 (interferon inducibilní protein 16). Tento protein patří do rodiny jaderných proteinů HIN-200, které mají pravděpodobně důležitou úlohu v transkripční regulaci. V rámci studia tohoto proteinu jsme publikovali krystalovou strukturu jeho dvou domén, Hin-A a Hin-B. Pomocí interakční analýzy proteinů jsme zjistili, že doména HIN-A proteinu IFI16 interaguje s C-

-terminální doménou proteinu p53. Pomocí gelové retardační analýzy jsme poté ukázali, že interakce proteinu IFI16 zlepšuje vazebné vlastnosti proteinu p53. Mutace domény HIN-A ruší aktivaci proteinu p53. IFI16 protein také zvyšuje transkripční aktivaci zprostředkovanou proteinem p53, jak jsme demonstrovali kotransfekcí konstruktů pro tvorbu p53 a proteinů IFI16 s luciferázovým reportérovým systémem s cílovým místem p53. Protein IFI16 je také schopný vázat se přímo na DNA s výraznou preferencí pro křížové struktury a superhelikální DNA. Interakce s proteinem p53 tedy může vést také k efektivnějšímu vyhledávání strukturně afinitních míst v DNA.

3. Závěr

Předkládaná habilitační práce shrnuje poznatky získané v oblasti vazby proteinů na superhelikální DNA a křížové struktury. Výsledky byly publikované v 17 článcích v impaktovaných a rezenzovaných zahraničních časopisech. Kromě toho kandidát publikoval i celou řadu dalších originálních výsledků.

V habilitační práci jsou nejprve shrnuty poznatky o lokálních strukturách, které se v DNA tvoří zejména se zaměřením na křížové struktury a jejich význam a přítomnost v genomu. Dále jsou podrobně popsány proteiny, které se váží na křížové struktury s jejich potenciálním významem u lidských onemocnění. Výsledky výrazně přispěly k poznání způsobu regulace vazby proteinu p53 a některých dalších proteinů k DNA. Protein p53 se váže na celou řadu cílových sekvencí v genomu a reguluje tak ochranné mechanismy působící po poškození buňky. Strukturní motivy pak hrají důležitou úlohu v efektivní účinnosti vazby na DNA. Vazba ke křížovým strukturám byla u proteinu p53 charakterizována několika metodikami a ukázalo se dokonce, že cílová místa pro protein p53 velmi často obsahují inverzní repetice a jsou schopná tvorby křížových struktur, což výrazně přispívá k intenzivní vazbě proteinu p53 k DNA. Pomocí chromatinové imunoprecipitace byla také ukázána preferenční vazba tohoto proteinu k sekvencím s inverzní repeticí v regulačních oblastech promotoru p21. Tato preference může hrát jednu z klíčových úloh při determinaci buňky k zastavení buněčného cyklu a apoptóze. Při větším genotoxickém zásahu dochází totiž nejprve k selektivnímu poškození aktivních míst, které jsou spojeny s extruzí křížové struktury. Tyto výsledky podporuje i specifická vazba proteinů 14-3-3 a IFI16 k superhelikální DNA a křížovým strukturám. Bylo také ukázáno, že protein IFI16 se může podílet i přímo na aktivaci proteinu p53.

Detailní charakterizace DNA vazebných vlastností proteinů, které s DNA interagují, je důležitým předpokladem porozumění základním biologickým procesům.

4. Abstract

Presented habilitation thesis summarizes results about the binding of proteins to supercoiled DNA and cruciform structures. The results were published in 16 articles in prestigious international journals. In addition, candidate published a number of other original results.

The habilitation thesis summarizes knowledge about local DNA structures with a particular focus to cruciforms and their importance and presence in the genome. There are sumarized informations about proteins which bind to cruciforms and their potential importance in human disease. The results contributed significantly to the understanding of the p53 binding to DNA and its regulation. P53 binds to a number of target sequences in the genome and thus regulates the protective mechanisms upon cell damage. Structural motifs play important role in the effective efficiency of binding to DNA. Binding to cruciform structures by the p53 protein is characterized by several methodological approaches. Moreover it was showed that the target sites for the p53 protein frequently contain inverted repeats and are capable to form cruciforms, which significantly contributes to the intensity of p53 binding to DNA. By using chromatin immunoprecipitation was also demonstrated preferential binding of this protein to sequences with inverse repeats in regulatory regions of the p21 promoter. This preference may play one of the key roles in the cell determination to cell cycle arrest and apoptosis. These results are also supported by specific binding proteins 14-3-3 and IFI16 to supercoiled DNA and cruciforms. It was also shown that the protein IFI16 also contribute directly to the activation of p53.

Detailed characterization of the DNA binding properties of proteins that interact with DNA is fundamental in understanding of basic biological processes.

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6. Seznam zkratek

BLM, Bloom syndrome protein

BMVC, 3,6-bis(1-methyl-4-vinylpyridin

BRCA1, breast cancer type 1 susceptibility protein

CNBP, cellular nucleic-acid-binding protein

Dna2, DNA replication helicase/nuclease 2

FANCJ, Fanconi anemia complementation group J

FMR2, fragile X mental retardation 2

G4R1, G4 Resolvase 1

hnRNP, heterogeneous nuclear ribonucleoprotein;

IGF-2, Insulin-like growth factor 2

MAZ, myc-associated zinc-finger

MLL, mixed lineage leukemia

PARP-1, Poly [ADP-ribose] polymerase 1

POT1, protection of telomeres 1

RHAU, the RNA helicase associated with AU-rich element

RPA, replication protein A

SAF, scaffold attachment factor

scDNA - supercoiled DNA (nadšroubovicová DNA)

Sgs1, small growth suppressor 1

SRSF, serin/arginine-rich splicing factor

TEBP, Telomere End Binding Protein

TLS/FUS, translocated in liposarcoma/fused in sarcoma

TMPyP4, tetra-(N-methyl-4-pyridyl)porphyrin

Topo I, Topoisomerase I

TRF2, telomere repeat binding factor 2

UP1, unwinding protein 1

WRN, Werner syndrome ATP-dependent helicase.

7. Seznam publikací a přílohy

Seznam publikací přiložených k habilitační práci (přílohy 1-16)

Příloha 1: Pospisilova, S., Brazda, V., Kucharikova, K., Luciani, M.G., Hupp, T.R., Skladal, P., Palecek, E., and Vojtesek, B. (2004). Activation of the DNA-binding ability of latent p53 protein by protein kinase C is abolished by protein kinase CK2. Biochem J 378, 939-947.

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Příloha 3: Brazda, V., Jagelska, E.B., Fojta, M., and Palecek, E. (2006). Searching for target sequences by p53 protein is influenced by DNA length. Biochem Biophys Res Commun 341, 470-477.

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Příloha 7: Palecek, E., Brazdova, M., Brazda, V., Palecek, J., Billova, S., Subramaniam, V., and Jovin, T.M. (2001). Binding of p53 and its core domain to supercoiled DNA. Eur J Biochem 268, 573-581.

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Activation of the DNA-binding ability of latent p53 protein by protein kinase C is abolished by protein kinase CK2

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p53 is one of the most important regulators of cell proliferation and differentiation and of programmed cell death, triggering growth arrest and/or apoptosis in response to different cellular stress signals. The sequence-specific DNA-binding function of p53 protein can be activated by several different stimuli that modulate the C-terminal domain of this protein. The predominant mechanism of activation of p53 sequence-specific DNA binding is phosphorylation at specific sites. For example, phosphorylation of p53 by PKC (protein kinase C) occurs in undamaged cells, resulting in masking of the epitope recognized by monoclonal antibody PAb421, and presumably promotes steady-state levels of p53 activity in cycling cells. In contrast, phosphorylation by cdk2 (cyclin-dependent kinase 2)/cyclin A and by the protein kinase CK2 are both enhanced in DNA-damaged cells. We determined whether one mechanism to account for this mutually exclusive phosphorylation may be that each phosphorylation event prevents modification by the other kinase. We used non-radioactive electrophoretic mobility shift assays to show that C-terminal phosphorylation of p53 protein by cdk2/cyclin A on Ser^{315} or by PKC on Ser^{378} can efficiently stimulate p53 binding to DNA *in vitro*, as well as binding of the monoclonal antibody Bp53-10, which recognizes residues 371–380 in the C-terminus of p53. Phosphorylation of p53 by CK2 on Ser^{392} induces its DNA-binding activity to a much lower extent than phosphorylation by cdk2/cyclin A or PKC. In addition, phosphorylation by CK2 strongly inhibits PKC-induced activation of p53 DNA binding, while the activation of p53 by cdk2/cyclin A is not affected by CK2. The presence of CK2-mediated phosphorylation promotes PKC binding to its docking site within the p53 oligomerization domain, but decreases phosphorylation by PKC, suggesting that competition between CK2 and PKC does not rely on the inhibition of PKC–p53 complex formation. These results indicate the crucial role of p53 C-terminal phosphorylation in the regulation of its DNA-binding activity, but also suggest that antagonistic relationships exist between different stress signalling pathways.

Key words: activation, cdk2/cyclin A, DNA binding, electrophoretic mobility shift assay (EMSA), p53, phosphorylation.

INTRODUCTION

The p53 tumour suppressor protein (reviewed in [1,2]) is a potent transcription factor, playing a key role in cell cycle regulation and differentiation. p53 protein is activated in response to a variety of cellular stress signals, including DNA damage, hypoxia, metabolic changes, heat shock, pH changes and oncogene activation, and triggers cell cycle arrest and/or apoptosis to prevent cells from undergoing tumorigenic alterations [3]. The p53 protein has been structurally and functionally divided into four domains, two of which are involved in its DNA-binding function. The central region, known as the core domain (residues 102–290 in human p53), is responsible for DNA binding in a sequence-specific manner, whereas the C-terminal negative regulatory domain (residues 364–393) is necessary for sequence-independent binding and regulates the binding capability of the core domain [4].

Wild-type p53 protein is structurally flexible, and can reversibly adopt a latent conformation, which does not bind DNA, or an active conformation, which binds DNA in a sequence-specific manner. The activation of latent p53 to enable it to bind DNA can be induced by events that target the C-terminal regulatory domain, e.g. by deletion of the last 30 amino acids, or by interaction with specific peptides [5,6], short single-stranded DNAs [7] or monoclonal antibodies [8,9], that can influence both sequence-specific and non-specific DNA-binding functions [10]. The regulatory mechanism of core domain sequence-specific DNA binding via the C-terminal domain involves the ability of the C-terminus to destabilize folding of the core DNA-binding domain [11]. Phosphorylation of the C-terminus neutralizes this destabilizing effect and prevents core domain unfolding, thus activating DNA binding [12]. After DNA binding is activated, p53 can be acetylated in a DNA-dependent manner by the transcriptional co-activator p300 [13]. This acetylation clamps p300 to the p53–DNA complex and presumably enhances recruitment of chromatin remodelling factors. Further, a post-DNA-binding role for acetylation was shown recently by the demonstration that the proline-repeat domain binds directly to p300 and that this binding overcomes the conformational constraint to acetylation in the p53 tetramer [14]. Thus a phosphorylation and acetylation cascade can play a role in activating DNA binding and in clamping p300 to the p53–DNA complex.

Abbreviations used: ATM, ataxia telangiectasia mutated; cdk, cyclin-dependent kinase; Chk, checkpoint kinase; DNA-PK, DNA-dependent protein kinase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GOPS, glycidoxypropyltrimethoxysilane; MDM2, murine double min clone 2 oncoprotein; PKC, protein kinase C.

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Thus, critical events for p53 protein stabilization and activation in response to cellular stress are post-translational modifications, including phosphorylation, acetylation, glycosylation [15] or conjugation with the small ubiquitin-like protein SUMO-1 (small ubiquitin-related modifier-1) [16]. p53 is phosphorylated by different cellular kinases on several serine and threonine residues within the N- and C-terminal regions [17].

The protein kinase CK1 phosphorylates Ser^{6} and Ser^{9} of p53 *in vitro*, and DNA-PK (DNA-dependent protein kinase) and ATR (ataxia telangiectasia-related) kinase phosphorylate Ser^{15} and Ser^{37}. Phosphorylation at Ser^{15}, which is important for transactivation properties and protein stability, is also mediated by ATM (ataxia telangiectasia mutated) kinase in response to UV- and ionizing radiation-mediated damage. The checkpoint kinases Chk1 and Chk2 can phosphorylate Thr^{18} and Ser^{20} by an allosteric activation step involving Chk2 docking to the DNA-binding domain of p53 [18]. This phosphorylation stabilizes p300 docking to the phosphorylated p53 transactivation domain and promotes the DNA-dependent acetylation of p53 [14,19]. Ser^{33} is phosphorylated by JNK (c-Jun kinase N-terminal kinase) and by cdk7/cyclin H/p36 kinase [20,21]. The N-terminal modifications contribute to p53 regulation by affecting the interaction of p53 with the negative regulatory protein MDM2 (murine double min clone 2 oncoprotein) and with transcriptional co-activators [22].

Several sites of potential modification are clustered within the C-terminus of human p53 and are important for its regulatory function. Lys^{320}, Lys^{373} and Lys^{382} are modified by acetylation with PCAF (p300/CBP-associated factor) and p300/CBP [CREB (cAMP response element-binding protein)-binding protein] [23], and although these modifications have been shown to enhance the DNA-binding activity of p53 *in vitro*, acetylation is conformationally constrained and is a post-DNA-binding event *in vivo* [13,19]. Ser^{315} of p53 is targeted by cdk1 and cdk2 (cyclin-dependent kinases 1 and 2). Ser^{378} is phosphorylated by PKC (protein kinase C) [24], and its *in vitro* dephosphorylation by phosphatases 1 and 2A leads to the restoration of reactivity with monoclonal antibody PAb421 and regeneration of p53 latency [25]. In contrast with Ser^{378}, which was shown to be phosphorylated in irradiated cells, Ser^{376} is dephosphorylated in the presence of ATM as a response to genotoxic stress, resulting in the association of p53 with the 14-3-3 adaptor protein [26]. PKC subunits α and ζ were also shown to phosphorylate Ser^{371} *in vitro* [27]. The experimental evidence that modification of p53 by PKC can be responsible for the induction of the G_1/S growth arrest of transformed cells harbouring a functional p53 allele relies on the observation that the PKC activator PMA can induce this G_1/S growth arrest [27a,27b]. Although these data provide evidence that PKC is involved in the activation of p53 in cells, it does not distinguish between the 12 existing isoforms of PKC, nor other enzymes that may potentially modify serine residues at these sites. The supporting evidence for a role of calcium- and diacylglycerol-dependent PKC isoforms in p53 modification in cells comes from reports describing activation of PKC using stress stimuli, such as UV-C radiation and gamma radiation, that can also activate the p53 pathway [28,29]. Ser^{392} is phosphorylated by the protein kinase CK2 after both UV and ionizing radiation treatment *in vitro* and *in vivo* [30,31]. Phosphorylation on Ser^{392} has been shown to enable the transcriptional activation of the p53 protein *in vitro* [5] and also seems to be important for p53-mediated transactivation *in vivo* [32]. CK2 is composed of two catalytic subunits, α and α', and two β regulatory subunits, and regulates p53 activity not only by phosphorylation on Ser^{392} but also by binding of the β subunits to the p53 oligomerization domain (residues 325–344). The β subunits were also shown to influence the DNA-binding activity of p53 [33] and, vice versa, wild-type p53 can inhibit CK2 kinase activity [34]. The region where the CK2 β subunit binds to p53 also functions as a docking site for several other kinases, including cdks and PKC [35,36] (Figure 1).

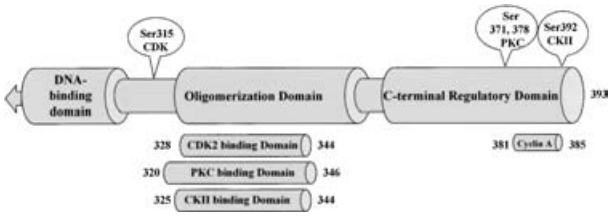

Figure 1 Key phosphorylation sites of human p53 protein and their localization within the protein's functional domains, and binding sites of protein kinases targeting the C-terminus of p53

The importance of different C-terminal modifications for the activation of the sequence-specific DNA binding of the latent form of p53 has still not been successfully explained, namely binding to response elements localized within the long DNA molecules. Here we have studied the mechanisms of p53 protein activation by various C-terminal domain phosphorylations, the role of the C-terminal-directed kinases CK2, PKC and cdks in this process and their relationship with respect to p53 protein binding. The fact that cdk2/CK2 and PKC exhibit mutually exclusive phosphorylations of p53, depending on the level of DNA damage, provides evidence that such a mechanism may operate *in vivo* and form a biochemical basis for the effects observed.

EXPERIMENTAL

Expression of p53 protein in bacteria and in insect cells

Human p53 protein was expressed in *Escherichia coli* DH5α cells carrying the pT7-7 (p53) plasmid, by isopropyl β-D-thiogalactoside induction (0.5 mM for 4 h), or in Sf9 insect cells infected with recombinant baculovirus. Bacterial cells were harvested and lysed according to the standard protocol. Sf9 cells were grown in suspension at 27 °C in Ex-cell 400 medium (JRH Bioscience) with 5 % (v/v) fetal bovine serum and 2 mM glutamine. At 3 days after baculovirus infection, Sf9 cells were washed twice in cold PBS, scraped, pelleted by centrifugation at 300 ***g*** for 5 min and lysed in the lysis buffer for 30 min on ice.

Purification of p53 protein

Cell lysates (from bacteria or insect cells) were centrifuged at 15 000 ***g*** for 30 min and the supernatant was diluted 5-fold in low-salt purification buffer [15 % (v/v) glycerol, 15 mM Hepes/KOH, pH 8.0, 0.04 % Triton X-100, 5 mM DTT (dithiothreitol), 2 mM benzamidine and 1 mM β-glycerophosphate], filtered and loaded on to a 5 ml heparin–Sepharose column (Amersham Biosciences). The p53 protein was eluted by a 0–1 M KCl gradient, and the peak fractions that eluted at approx. 0.5–0.6 M KCl were pooled, dialysed against low-salt purification buffer for 12 h at 4 °C and loaded on to an anion exchange HQ column of the BioCad Sprint perfusion chromatography system (PerSeptive Biosystems, Inc.).

Expression and purification of cdk2 and cyclin A

Human cdk2 and cyclin A were expressed in Sf9 insect cells using recombinant baculoviruses (kindly provided by Dr K. Ball, Cancer Research UK Laboratories, Department of Surgery and Molecular Oncology, University of Dundee Medical School, Dundee, U.K.) grown in suspension at 27 °C in Ex-cell 400 medium (JRH

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Bioscience) containing 5 % (v/v) fetal bovine serum and 2 mM glutamine. Each recombinant protein was purified according to the methods described in [36].

In vitro phosphorylation of p53 protein

Purified bacterial p53 protein was phosphorylated *in vitro* by CK2 (New England Biolabs), PKC (Promega), calcium- and diacylglycerol-dependent PKC consisting of α, β and γ isoforms, DNA-PK (New England Biolabs) or cdk2 complexed with cyclin A, expressed in the baculoviral system. Purified glutathione S-transferase–Chk1 fusion protein was a gift from Dr J. Hutchins (University of Dundee) [37]. The kinase reaction mixture contained 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM $CaCl_2$, 100 μg/ml phosphatidylserine, 20 μg/ml dioleoyl-*sn*-glycerol, 1 mM CHAPS and 0.2 mM ATP, and the reaction was performed at 30 °C for 30 min. Samples were analysed by Western blotting and by EMSA (electrophoretic mobility shift assay) for DNA-binding activity.

In vitro radiolabelling of p53 protein

The purified bacterial p53 protein was phosphorylated *in vitro* as described above by the addition of [γ-^{32}P]ATP (37 kBq; 1 μCi; Amersham Biosciences). The kinase reaction was performed at 30 °C for 30 min and analysed by SDS/PAGE on 10 % (w/v) polyacrylamide gels. The gels were stained, dried and analysed by phosphorus screen autoradiography on a STORM optical scanner (Molecular Dynamics). The level of phosphorylation was evaluated quantitatively using densitometric tracing (ImageQuant; Molecular Dynamics).

Western-blot analysis and antibodies

The protein was analysed using SDS/10 %-PAGE according to the standard protocol. Monoclonal antibodies were purified from ascites using Protein A columns. The final antibody concentration, as determined by Bradford protein assay, was 1 μg/ml in all cases. The antibodies used in this study were as follows. (a) DO-1 monoclonal antibody, directed towards the epitope S^{20}DLWKL^{25} within the p53 N-terminal region [38]. (b) CM1 polyclonal antibody, which recognizes many epitopes on the human p53 protein [39]. (c) PAb421 monoclonal antibody, which recognizes the epitope S^{371}KKGQSTSRH^{380} within the p53 C-terminal region [40] and displays a strong preference for the non-phosphorylated epitope [9]. (d) FPS315 phospho-specific monoclonal antibody, which recognizes p53 phosphorylated on Ser^{315} [41]. (e) S-P-3 phospho-specific monoclonal antibody, which recognizes the C-terminus of p53 protein phosphorylated on Ser^{392} [42]. (f) Ica9 monoclonal antibody, which recognizes the epitope E^{388}GPDSD^{393} within the p53 C-terminal region [43] and displays a strong preference for the non-phosphorylated epitope [5].

Sequence-specific DNA probes

For radioactive EMSAs, we used 20 bp oligonucleotides containing a single p53 recognition sequence (AGACATGCCTAGACATGCCT), and a p53 non-recognition sequence (GCATCATAGCGCATCATAGC) as a negative control. Complementary oligonucleotides were hybridized and end-labelled with [γ-^{32}P]ATP using T4 kinase. In non-radioactive EMSAs, we used the plasmid pPGM1 (2987 bp; derived from pBluescript II SK) containing one copy of the 20-mer consensus sequence (AGACATGCCTAGACATGCCT) cloned into the *Hin*dIII restriction site [44]. As a negative control, we used the plasmid pBluescript II SK (2961 bp). Both plasmids were digested with the restriction endonuclease *Pvu*II (MBI Fermentas), producing two restriction fragments: 2513 bp without the consensus sequence and 474 bp containing the consensus sequence (from pPGM1), or 448 bp without the consensus sequence (from pBluescript II SK).

EMSA using PAGE

p53 protein (100 ng) was diluted in activation buffer (5 mM Tris/HCl, pH 7.8, 0.5 mM EDTA, 50 mM KCl, 0.01 % Triton X-100). Then 5 ng of radiolabelled oligonucleotide with (or without) the p53 consensus sequence was added to the reaction mixture and incubated on ice for 30 min. Samples were then analysed by native PAGE on 4 % gels in 0.5 × TBE buffer (89 mM Tris/borate, 2 mM EDTA) at 200 V for 120 min at 4 °C.

EMSA using agarose gel electrophoresis

p53 protein (100 ng) was diluted in activation buffer (as above) and incubated for 30 min at 30 °C with 2 μg of specific monoclonal antibody. A 1 μg sample of DNA restriction fragment, with or without the p53 consensus sequence, was added to the reaction mixture and incubated on ice for 30 min. Loading buffer was added to the reaction mixture and samples were immediately loaded on to a 1.2 % native agarose gel. Electrophoresis was performed in TBE buffer at 10 V/cm for 200 min at 4 °C. After electrophoresis, gels were stained with ethidium bromide (1 μg/ml) for 30 min, rinsed in distilled water, photographed and evaluated quantitatively using densitometric tracing.

Measurement of protein–protein interactions using the piezoelectric biosensor

Piezoelectric biosensor methodology was used to study the interaction of p53 with kinases in real time. The biosensor reflects the changes in the resonant frequency of the crystal bearing the covalently immobilized p53 protein, which are directly proportional to the amount of biomolecules bound to the crystal surface. Piezoelectric crystals (10 MHz, AT cut, gold electrodes) were obtained from International Manufacturing Company (Oklahoma City, OK, U.S.A.). For immobilization of p53 protein, the crystals were washed carefully with acetone and activated further with GOPS (glycidoxypropyltrimethoxysilane) according to the standard protocol. The crystals were incubated for 1 h in 10 % (w/v) GOPS solution (in 95 % ethanol) at 20 °C and dried for 6 h at 50 °C. The GOPS-modified crystal was inserted into the flow-through cell (internal volume 40 μl), connected to the oscillator circuit and the output frequency was determined using a UZ 2400 counter (UTES/Grundig, Brno, Czech Republic). The resonant frequency of the crystal was recorded and displayed using a custom LabTools program in MS Windows. The cell was connected to a Minipuls MP3 peristaltic pump (Gilson, Villeurbanne, France), silicone tubes (internal diameter 0.16 mm) were used for all connections, the flow rate was kept constant at 25 μl/min and all experiments were carried out at 20 °C. Purified p53 protein was added to the flowing solution at a concentration of 5 μg/ml and allowed to bind for 15 min in the presence of 50 mM phosphate buffer containing 1 mM DTT, pH 9.5. Crystals with covalently immobilized protein p53 thus obtained were used for further experiments. The carrier buffer was 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 1 mM DTT. To study interactions of p53-modified crystals with protein kinases, solutions of either CK2 or PKC dissolved in the carrier solution were allowed to interact with the crystals; when indicated, 0.2 mM ATP was present in the solution. The binding curve was recorded for 8–10 min, and then the dissociation of the formed p53–kinase affinity complex was determined in the presence of carrier buffer only.

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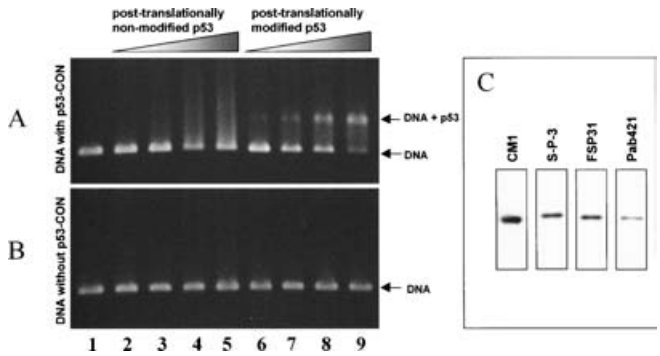

Figure 2 DNA-binding activity of post-translationally modified and non-modified p53 protein

Increasing amounts of post-translationally modified p53 expressed in Sf9 insect cells or bacterially expressed non-modified p53 were incubated with either (**A**) the pPGM1 plasmid carrying the p53 consensus sequence (p53-CON; 474 bp fragment) or (**B**) the pBluescript plasmid lacking the p53 consensus sequence (448 bp fragment). The reaction was performed in the presence of a linear plasmid DNA fragment as a non-specific competitor. The DNA–p53 complexes were separated by electrophoresis on a 1.2 % (w/v) agarose gel as described in the Experimental section. Lane 1, DNA fragment only (250 ng); lanes 2–5, bacterial p53 protein (0.25, 0.5, 1.0 and 1.5 μg respectively), lanes 6–9, baculoviral p53 protein (0.25, 0.5, 1.0 and 1.5 μg respectively). (**C**) p53 expressed in Sf9 insect cells was subjected to 10 % denaturing PAGE. Specific phosphorylation was detected by immune Western blotting using CM1 antibody and the indicated phospho-specific monoclonal antibodies.

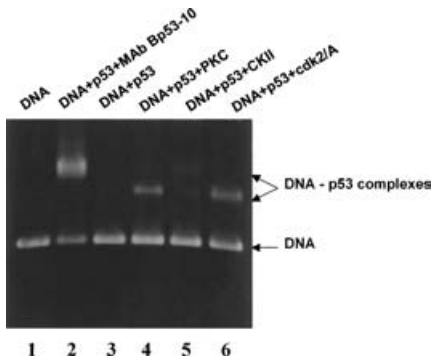

Figure 3 Activation of sequence-specific DNA binding of latent p53 protein by monoclonal antibody binding and phosphorylation

Samples of 100 ng of bacterially expressed p53 (non-modified, phosphorylated *in vitro* or bound with C-terminal anti-p53 monoclonal antibody Bp53-10) were incubated with a 474 bp fragment of the pPGM1 plasmid carrying the p53 consensus sequence. EMSA for DNA–p53 complex detection was performed in a 1.2 % agarose gel. Lane 1, DNA pPGM1/*Pvu*II (fragment of 474 bp carrying the p53 consensus sequence); lane 2, DNA + p53 protein (bacterial) + activating monoclonal antibody (MAb) Bp53-10; lane 3, DNA + p53 (bacterial); lane 4, DNA + p53 phosphorylated by PKC; lane 5, DNA + p53 phosphorylated by CK2 (CKII); lane 6, DNA + p53 phosphorylated by cdk2/cyclin A.

RESULTS

Binding of non-modified and post-translationally modified p53 to DNA fragments containing the consensus response element

The p53 tumour suppressor protein is known to be regulated via C-terminal phosphorylation by cdk2, PKC and CK2. In order to investigate the differential phosphorylation of p53 by these kinases and their effects on the DNA-binding activity of p53, we used the EMSA technique to show the extent of p53 protein–DNA interactions, together with piezoelectric sensor measurements to detect p53 protein–kinase interactions.

The DNA-binding activity of post-translationally modified (expressed in Sf9 insect cells) and non-modified (expressed in *E. coli*) p53 protein using agarose gel EMSA is shown in Figure 2. The post-translationally modified protein (the 'active' fraction phosphorylated on Ser^{315}, Ser^{378} and Ser^{392} and recognized by phospho-specific monoclonal antibodies FPS315 and S-P-3, but partially non-recognized by PAb421, as detected by immunoblotting in Figure 2C) displayed significant sequence-specific DNA-binding activity, resulting in the formation of a retarded band of the p53–DNA complex in the presence of non-specific linear DNA as a competitor. In contrast, the non-post-translationally modified p53 protein did not show any sequence-specific DNA binding (Figure 2A). The same DNA restriction fragment, but without a p53 consensus response element, was used as a negative control (Figure 2B) to demonstrate the specificity of the reaction. Analogous data have been obtained previously in EMSAs of oligonucleotide p53 response elements and by DNase I footprinting, highlighting the importance of post-translational protein modifications for the sequence-specific DNA-binding properties of p53.

Activation of the sequence-specific DNA binding ability of p53 protein by C-terminal monoclonal antibodies and C-terminal phosphorylation

The sequence-specific DNA-binding activity of latent p53 protein can be stimulated by C-terminal-specific monoclonal antibodies such as PAb421 (recognizing an epitope comprising residues 371–380), Bp53-6 (epitope 381–390) and Bp53-10 (epitope 371–380). The previously reported activation of latent p53 protein to a form that binds DNA by monoclonal antibody Bp53-10 is shown in Figure 3 (lane 2), where the retarded band of the p53–DNA–antibody complex is shown. Here we show that phosphorylation of the C-terminal part of p53 protein could also activate its DNA-binding function and induce p53 sequence-specific binding not only to oligonucleotides but also to consensus sequences localized within longer DNA molecules. The retarded band of the p53–DNA complex occurred after phosphorylation by PKC on Ser^{378} or by cdk2/cyclin A on Ser^{315} (Figure 3, lanes 4 and 6); phosphorylation by CK2 on Ser^{392} resulted in only very low activation of p53 that was hardly detectable by EMSA (Figure 3, lane 5). The phosphorylation of the N-terminal domain of p53 by DNA-PK or Chk1 did not influence the DNA-binding activity of p53 (results not shown). Kinase reactions performed without the addition of ATP, which provided the best negative control, did not result in any activation of DNA binding, suggesting that protein phosphorylation was responsible for this effect.

The efficiency of *in vitro* phosphorylation was demonstrated by radiolabelling of p53 using [γ-^{32}P]ATP. This method showed that phosphorylation of the p53 protein reached an equilibrium with all kinases used (i.e. cdk2/cyclin A, CK2 and PKC) within 30 min of kinase reaction. The equilibrium was not due to kinase inactivation, but to the saturation of the p53 phosphorylation sites, as indicated by the following: (a) addition of kinase to the reaction for a second incubation (e.g. for another 30 min) did not increase the level of p53 phosphorylation, and (b) addition of non-modified p53 protein to the reaction mixture for a further 30 min proportionally increased the ^{32}P response, confirming the stability of the kinases. These data allowed us to conclude that all three phosphorylation sites (Ser^{378}, Ser^{315} and Ser^{392}) were modified stoichiometrically. To verify this assumption further, we performed Western blot analysis with two monoclonal antibodies, S-P-3 and FPS315, which recognize phosphorylated (but not unphosphorylated) Ser^{392} or Ser^{315} respectively. With both antibodies we observed strong bands after a 15 min incubation with the respective kinase – CK2 or cdk/A (Figures 4A and 4B) – on our Western blots. The intensity of these bands was not changed after a

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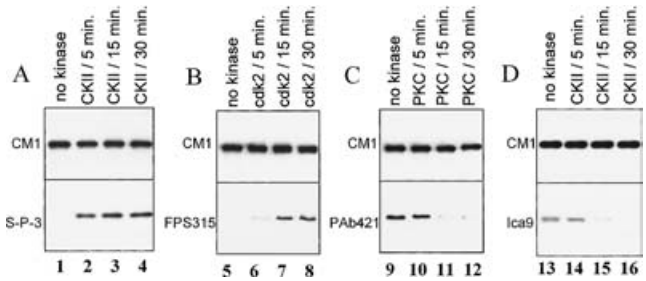

Figure 4 Detection of p53 C-terminal phosphorylation using phospho-specific monoclonal antibodies

Bacterially expressed p53, phosphorylated *in vitro* by CK2, PKC or cdk2/cyclin A, was subjected to 10 % denaturing PAGE. Specific phosphorylation was detected by immune Western blotting using phospho-specific monoclonal antibodies. Lanes 1–4, CK2 (CKII)-phosphorylated p53 detected with S-P-3 (specific for p53 phosphorylated on Ser^{392}); lanes 5–8, cdk2/cyclin A-phosphorylated p53 detected with FPS315 (specific for p53 phosphorylated on Ser^{315}); lanes 9–12, PKC-phosphorylated p53 detected using PAb421 (preferentially recognizes p53 protein not phosphorylated on Ser^{378}); lanes 13–16, CK2-phosphorylated p53 detected with Ica9 (preferentially recognizing p53 protein not phosphorylated on Ser^{392}). Kinase reactions were performed for 5, 15 and 30 min as indicated.

30 min incubation, similar to the results of ^{32}P radiolabelling. It is well known that phosphorylation may occur on a residue that is a part of an epitope for a particular monoclonal antibody and that this may result in a decrease in immunoreactivity, to the extent that the antibody fails to bind to the phosphorylated target protein. For these reasons, phosphorylation of p53 at Ser^{392} was analysed additionally using Ica9 antibody, which prefers binding to the non-phosphorylated epitope $E^{388}GPDS^{393}$ of p53. Our results show (Figure 4D) that, after a 15 min incubation of p53 with CK2, almost all p53 was phosphorylated, and after a 30 min interval there was no non-phosphorylated epitope available for antibody Ica9. The intensity of the band with the control CM1 polyclonal antibody, which recognizes many epitopes of both phosphorylated and non-phosphorylated p53 protein, was not influenced by kinase treatment (Figure 4). p53 phosphorylation by PKC was monitored by the PAb421 antibody, which also preferentially recognizes p53 only when not phosphorylated on Ser^{378}. After a 15 min incubation of p53 with PKC, the PAb421 band almost disappeared (Figure 4C), suggesting that almost all Ser^{378} was phosphorylated. The above data thus confirmed our assumption that practically all serine residues were phosphorylated under the given conditions (Figure 4) by each of the three kinases used in our experiments.

Figure 5(A) shows the phenomenon of p53 activation by C-terminal phosphorylation in more detail. Sequence-specific DNA-binding activity of p53 was mainly stimulated by cdk2/cyclin A and by PKC, whereas phosphorylation by CK2 did not have such a marked effect, as shown by the weak intensity of the retarded band. In addition, CK2 phosphorylation-induced p53–DNA complexes were significantly more retarded than cdk2/cyclin A- and PKC-phosphorylated p53–DNA complexes, suggesting that they have a higher molecular mass (Figure 5A, lanes 2–4; Figure 5B, lane 5). Incubation with monoclonal antibody DO-1 provided a supershift of the p53–DNA complexes and demonstrated the presence of p53 protein in the complex (Figure 5A, lanes 4, 8 and 12). A parallel experiment showed the binding of phosphorylated p53 to an oligonucleotide consensus response element (20-mer; AGACATGCCTAGACATGCCT) using PAGE (Figure 5B). Phosphorylation by all three kinases for 30 min resulted in the formation of a retarded band; in addition, the electrophoretic mobility of the CK2-phosphorylated p53–DNA complex was lower than that of those due to phosphorylation by PKC and cdk2/cyclin A.

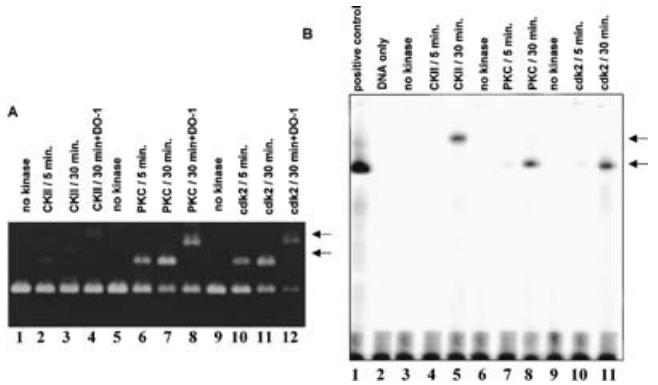

Figure 5 Activation of p53 by phosphorylation by CK2, PKC and cdk2/cyclin A

(**A**) Samples of 100 ng of bacterially expressed p53 protein were phosphorylated *in vitro* by CK2, PKC or cdk2/cyclin A, which target the C-terminal domain. Kinase reactions were performed for 5 or 30 min at 30 °C in the presence or absence of the anti-p53 monoclonal antibody DO-1. The DNA–p53 complexes were separated on a 1.2 % agarose gel as indicated by the arrows. Lanes 1, 5 and 9, non-modified p53 + consensus DNA; lanes 2 and 3, p53 phosphorylated by CK2 (CKII) (5 and 30 min respectively) + consensus DNA; lane 4, CK2-phosphorylated p53 (30 min) + consensus DNA + DO-1; lanes 6 and 7, PKC-phosphorylated p53 (5 and 30 min respectively) + consensus DNA; lane 8, PKC-phosphorylated p53 (30 min) + consensus DNA + DO-1; lanes 10 and 11, cdk2/cyclinA-phosphorylated p53 (5 and 30 min respectively) + consensus DNA; lane 12, cdk2/cyclin A-phosphorylated p53 (30 min) + consensus DNA + DO-1. (**B**) Samples of 100 ng of non-modified or *in vitro* phosphorylated p53 protein were incubated with radioactively labelled 20-mer DNA consensus sequence and the p53–DNA complexes were separated on 4 % non-denaturing PAGE and detected by autoradiography. The baculovirus-expressed p53 protein was used as a positive control (lane 1).

These results, which correlate well with the results obtained by agarose gel shift in Figure 5(A), suggest similar DNA-binding behaviour of the protein towards both oligonucleotides and long DNA molecules. The small quantitative difference between the proportion of CK2-induced DNA-binding activity of p53 to the oligonucleotide and to a 474 bp fragment indicates possible negative influences of non-specific DNA sequences on the sequence-specific DNA-binding activity of CK2-phosphorylated p53 protein.

Multiple phosphorylation of the C-terminus of p53 does not increase its DNA-binding activity significantly

The effects of multiple phosphorylation of p53 at C-terminal modification sites on its DNA-binding activity are presented in Figure 6. p53 was incubated with cdk2/cyclin A, PKC and CK2 either separately or in different combinations (CK2 + PKC, CK2 + cdk2, PKC + cdk2 and CK2 + PKC + cdk2), as indicated in Figure 6. We noted that: (a) phosphorylation by cdk2/cyclin A activated p53 protein more efficiently than did that by PKC or CK2, (b) double phosphorylation by cdk2/cyclin A and PKC did not result in a significant increase in p53 activity compared with that after phosphorylation with either kinase alone, (c) phosphorylation by CK2 was only a very weak activator of p53 DNA-binding activity, and (d) preincubation with CK2 inhibited the activatory effect of PKC, but not that of cdk2/cyclin A.

Phosphorylation by CK2 prevents PKC-induced p53 protein activation

To characterize further the phenomenon of competition between CK2 and PKC, which can play an important role in the co-operation of cell signalling processes, p53 was incubated in the presence of CK2, PKC or a combination of both kinases (Figure 7). The retarded bands in lanes 2 and 3 show p53 activation

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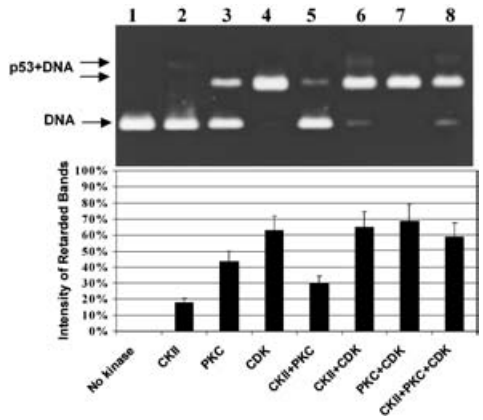

Figure 6 Kinase co-operation and competition during p53 phosphorylation

Samples of 100 ng of bacterially expressed p53 protein were phosphorylated *in vitro* with CK2, PKC, cdk2/cyclin A or different combinations of these kinases added to the reaction at the same time. DNA–p53 complexes were separated by EMSA on a 1.2 % agarose gel. Lane 1, 474 bp pPGM1/*Pvu*II DNA fragment carrying the p53 consensus sequence + non-phosphorylated p53 protein; lane 2, DNA + CK2 (CKII)-phosphorylated p53; lane 3, DNA + PKC-phosphorylated p53; lane 4, DNA + cdk2/cyclin A-phosphorylated p53; lane 5, DNA + p53 phosphorylated with CK2 and PKC; lane 6, DNA + p53 phosphorylated by CK2 and cdk2/cyclin A; lane 7, DNA + p53 phosphorylated by PKC and cdk2/cyclin A; lane 8, DNA + p53 phosphorylated by CK2, PKC and cdk2/cyclin A. Lower panel: quantification of super-shifted DNA–p53 complexes by densitometric tracing (representative measurement of three independent experiments). The total intensity of all bands within each lane was taken as 100 %.

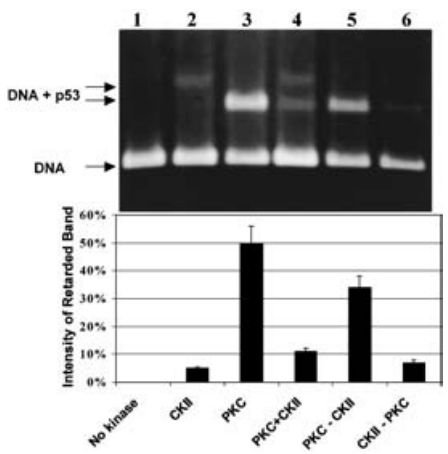

Figure 7 Competition between CK2 and PKC: the presence of CK2 prevents phosphorylation of p53 protein by PKC

Samples of 100 ng of bacterially expressed p53 protein were phosphorylated in the presence of CK2 (CKII) or PKC for 30 min either alone or in combination. When CK2 and PKC were used, they were added to the reaction either at the same time or subsequently (15 min after the beginning of the first kinase reaction). DNA–p53 complexes were separated by EMSA on a 1.2 % agarose gel. Lane 1, 474 bp pPGM1/*Pvu*II DNA fragment carrying the p53 consensus sequence + non-phosphorylated p53 protein; lane 2, DNA + CK2-phosphorylated p53; lane 3, DNA + PKC-phosphorylated p53; lane 4, DNA + p53 phosphorylated by CK2 + PKC (added simultaneously); lane 5, DNA + p53 protein preincubated with PKC (15 min) and then phosphorylated by CK2 (30 min); lane 6, DNA + p53 protein preincubated with CK2 (15 min) and then phosphorylated by PKC (30 min). Formation of the DNA–p53 complexes was indicated by super-shifted bands, and the intensity of the bands was quantified using densitometric tracing. Representative measurements of three independent experiments are shown. The total intensity of all bands within each lane was taken as 100 %.

induced by CK2- and PKC-mediated phosphorylation respectively. The sample in lane 4 was phosphorylated by both CK2 and PKC added to the reaction mixture at the same time. The intensity of the retarded band corresponding to the PKC-phosphorylated p53–DNA complex was significantly decreased (approx. 4-fold), suggesting an inhibitory effect of phosphorylation by CK2 on PKC-induced p53 activation. This was not observed if the p53 protein was preincubated with PKC for 15 min in the presence of 0.2 mM ATP prior to phosphorylation by CK2 (lane 5). In contrast, preincubation of p53 protein with CK2 and 0.2 mM ATP for 15 min prior to phosphorylation by PKC resulted in the complete abolition of p53 DNA-binding activity (lane 6). This effect was not observed in the absence of ATP. These results indicate a direct inhibitory effect of p53 phosphorylation by CK2 at Ser^{392} on the phosphorylation of human p53 by PKC. The kinase reactions performed in the absence of ATP did not stimulate p53 activity, demonstrating that phosphorylation rather than kinase binding or other conformational changes is responsible for p53 activation.

The inhibitory effect of the phosphorylation of p53 by CK2 on phosphorylation by PKC was also detected using radioactive ATP labelling, and similar results were obtained. Phosphorylation of p53 by CK2 and PKC when the kinase reactions were started at the same time led to a mild increase in total protein phosphorylation, as measured by the ^{32}P signal. However, preincubation of p53 with CK2 in the presence of [γ-^{32}P]ATP led to the complete abolition of p53 phosphorylation by PKC (the ^{32}P signal did not change further due to subsequent phosphorylation by PKC).

The kinetics of the binding of CK2 and PKC to p53 docking sites and the role of this interaction in their competition were studied further using the piezoelectric biosensor system. This system, a convenient tool for the characterization of bioaffinity interactions [45], reflects changes in the resonant frequency of a crystal which are directly proportional to the amount of biomolecules bound to the sensing surface, and enables the affinity interaction to be followed in real time. Here a decrease in the resonant frequency of a p53-modified crystal corresponds to the interaction of p53 with another molecule, i.e. CK2 or PKC dissolved in kinase buffer. The interactions of p53-modified crystals with these kinases were studied in the presence (Figure 8, left panel, curves b and d) or absence (curves a and c) of ATP. PKC easily bound to p53 (curves a and b), and addition of the carrier buffer resulted in the complete dissociation of PKC (the resonant frequency returned to its former level). Subsequent addition of CK2 induced partially irreversible binding of the kinase, which did not dissociate from the p53 protein. The CK2 β subunits that bind to the p53 oligomerization domain in the region between amino acids 325 and 344 (Figure 1) seem to be responsible for this effect. This finding also explains the results shown in Figures 3 and 5, where the CK2-phosphorylated p53 complexes with DNA displayed a lower electrophoretic mobility than the p53–DNA complexes induced by other kinases. Surprisingly, binding of CK2 β subunits to the docking site on p53 shared with several C-terminal kinases, including PKC, did not prevent PKC from binding to p53 in either the presence or the absence of ATP (Figure 8, left panel, curves c, d). In addition, phosphorylation of p53 by CK2 promoted the ability of PKC to bind to p53 (curve d), but not to phosphorylate the p53 protein, inducing its DNA-binding activity. The mass change due to phosphorylation of the immobilized p53 was too small to be visible by the piezoelectric biosensor. However, the effect of p53 phosphorylation by CK2 was clearly demonstrated by the subsequent 3-fold higher binding of PKC (curve d). From these results we can conclude that phosphorylation by cdk2/cyclin A and PKC, rather than by CK2, activates the latent ability of p53 to bind specific DNA sequences. Co-operation between different signalling pathways is mutually antagonistic, acting via a hierarchal mechanism, and probably has a profound influence on one of the crucial functions of the p53 protein, i.e. its DNA-binding activity.

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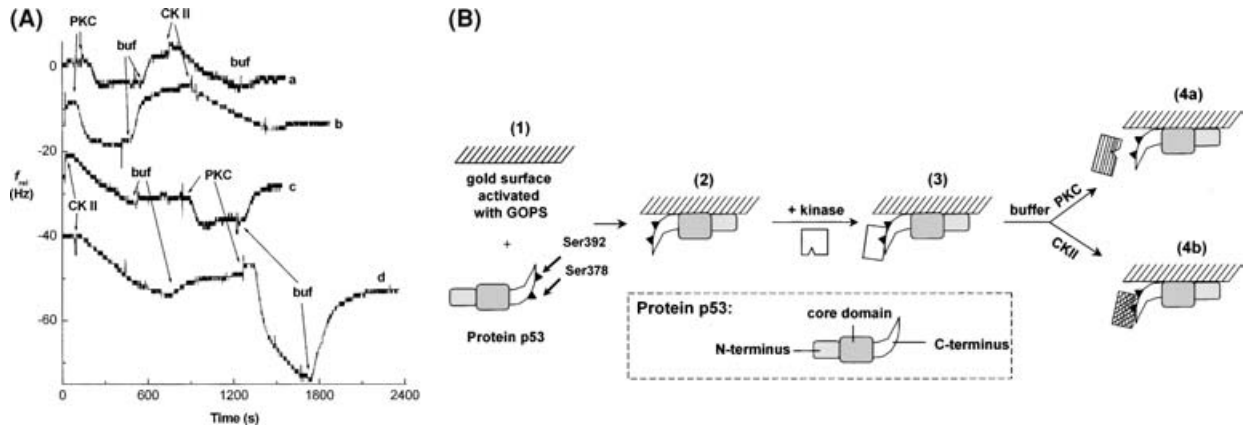

Figure 8 Interactions of p53 with kinases measured using a piezoelectric biosensor

(**A**) The p53-modified piezoelectric crystal was used in flow-through mode. Binding curves obtained during affinity interactions are shown as plots of relative frequency against time. The associations of p53 with either PKC or CK2 (CKII) are presented. Injection of kinases was carried out in either the absence (curves a, c) or the presence (curves b, d) of ATP (0.2 mM). Dissociation of affinity complexes formed at the sensing surface was also studied in the presence of carrier buffer only (buf). The individual traces have been vertically shifted for the sake of clarity. (**B**) Scheme of interactions of two kinases (CK2 and PKC) with p53 protein as measured by the piezoelectric sensor. Steps (1) and (2), p53 protein is covalently bound to the activated gold surface; (3), abundant PKC or CK2 kinases are added to the flow-through system, binding specifically to Ser^{378} or Ser^{392} respectively; this binding is reflected by a decrease in the resonant frequency; (4a), PKC molecules dissociate from p53 after the enzyme in solution is replaced by a blank buffer, as indicated by the return of the resonant frequency almost to its original value; (4b), with CK2 almost no changes in resonant frequency are observed, suggesting that this kinase remains bound to p53 under the same conditions.

DISCUSSION

Post-translational modifications have been shown to play a crucial role in the activity of the p53 protein. Higher levels of p53 phosphorylation and acetylation, together with specific p53 phosphorylation patterns, have been found in human tumour tissues [46]. p53 protein has two regions that are subject to multiple phosphorylations as well as other post-translational modifications (Figure 1). The N-terminal modifications influence the transactivation capabilities of p53 and regulate its interaction with the MDM2 oncoprotein. Modifications within the C-terminal region of p53 were suggested to play an important role in the regulation of its DNA-binding activity towards specific responsive elements, and the presence of an unmodified C-terminus was shown to prevent its specific transactivation function [47].

Several studies concerning the activation of p53 binding to consensus DNA sequences by C-terminal phosphorylation have been reported [5,25], mostly taking into account modifications by a single kinase. In contrast, here we directly compare for the first time the combined effects of phosphorylation by various kinases targeting the p53 C-terminal domain, such as CK2, PKC and cdk2 (either alone or in combination) on the activation of p53 DNA-binding to a specific consensus sequence. The binding sequences were presented not only in the form of oligonucleotides, but also localized in longer DNA molecules to reflect possible effects of neighbouring DNA sequences, including their conformation. Binding activity towards the consensus sequence was not observed in the case of p53 protein lacking post-translational modifications (Figure 2), which confirms the previously observed necessity for activation of the p53 transcription factor function [5]. Modified p53 protein expressed in a eukaryotic expression system provided retarded bands of protein–DNA complexes in EMSA gels. The non-radioactive EMSA technique that we used seems to be more convenient for these studies compared with the radioactive approach, as it enables more sensitive quantification of different protein–DNA complexes (Figures 5 and 6).

We have previously reported the ability of the C-terminus-specific monoclonal antibody Bp53-10 to activate the sequence-specific binding function of p53 [9,48]. Phosphorylation of the C-terminal region of p53 had the same effect as monoclonal antibody binding in an agarose EMSA gel, with the difference in the molecular size of the complexes being due to the presence or absence of the antibody molecule. Our results show that modification of Ser^{315}, residing just outside the oligomerization domain, has the strongest stimulatory effect on p53 activation. We have also detected phosphorylation of the same amino acid *in vivo* after UV irradiation of MCF7 and A375 cells, co-inciding with elevated p53-dependent transcription [41]. These findings suggest an important role for this pathway and for Ser^{315} phosphorylation in the stimulation of p53 function.

In contrast, we show that phosphorylation of Ser^{392} by CK2 stimulates the DNA-binding ability of p53 only very weakly. The first studies of this phenomenon [8] showed the *in vitro* activation of p53 DNA binding by purified CK2 from rabbit muscle, and also described the allosteric inhibition of the DNA-binding activity of p53 by the Ica9 antibody, which recognizes the CK2 target site [43]. There is also evidence that Ser^{392} phosphorylation increases the association constant for reversible tetramer formation nearly 10-fold, while phosphorylation of Ser^{315} and Ser^{378} had only a small effect on tetramer formation [49]. In contrast, results from Fiscella et al. [50] based on studies with the Ser^{392} mutant showed that neither phosphorylation of nor RNA attachment to Ser^{392} is required for the ability of p53 to suppress cell growth or to activate transcription *in vivo*, and that Ser^{392} phosphorylation has no discernible effect on p53 function. With respect to these discrepancies concerning the role of Ser^{392} phosphorylation in p53 protein activity, we attempted here to elucidate the role of CK2 in p53 function.

We also considered the possibility that not only Ser^{392} phosphorylation but also interaction of kinases with p53 protein could influence its activity. The CK2 regulatory β subunits have been shown to bind stably to the kinase docking region situated between amino acids 325 and 344 [51], which may explain the lower electrophoretic mobility of p53–DNA complexes phosphorylated by CK2 compared with complexes phosphorylated by other kinases (Figures 3 and 5). It has been shown not only that the

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stable interaction of CK2 β subunits with p53 has implications for p53 behaviour, but also that p53 influences the enzymic activity of CK2. The p53 C-terminus stimulates CK2 activity, but conversely full-length wild-type p53 inhibits the activity of CK2 [34]. The importance of CK2 for p53 protein activation therefore seems to be controversial. Thus we also concentrated on the effect of phosphorylation by CK2 on the modifications and activation of p53 protein by other C-terminal kinases. Results shown in Figures 6 and 7 suggest that the CK2-mediated phosphorylation of p53 has no effect on phosphorylation by cdk2, but strongly inhibits phosphorylation by PKC and subsequent stimulation of p53 DNA-binding activity. CK2 added to the kinase reaction mixture together with PKC, in the presence of ATP, significantly decreased the phosphorylation of p53 by PKC, and CK2 added 15 min before PKC completely abolished phosphorylation by PKC. These results were obtained both using the EMSA experiments shown in Figures 6 and 7 and by radioactive ATP phosphorylation measurements, where no changes in the phosphorylation level due to PKC were detected in case of p53 preincubation with CK2 in the presence of ATP. These findings can explain the results obtained by Schuster et al. [33], where co-expression of CK2 with p53 protein in mammalian cells inhibited the sequence-specific DNA binding of p53. We suggest that this inhibitory effect of CK2 is caused by the abolition of phosphorylation by PKC, which is a strong activator of the DNA-binding function of p53 (Figures 3 and 5).

In order to test whether CK2 prevents PKC from binding to its docking site on p53 or whether it allows PKC binding but prevents PKC-mediated phosphorylation, we developed a new binding assay based on piezoelectric biosensors, which are able to detect very sensitively the presence of kinase–p53 complexes. Using this approach we found that binding and phosphorylation of p53 by PKC did not affect the binding or phosphorylation of p53 by CK2 (PKC molecules dissociated completely from p53). In contrast, CK2 bound irreversibly to p53 in the presence or absence of ATP, but surprisingly this interaction did not influence PKC binding to p53, in spite of the docking site being common to both kinases. In addition, the presence of CK2 phosphorylation promoted the binding of PKC to p53 (Figure 8). Nevertheless, phosphorylation by PKC was dramatically blocked in the presence of phosphorylation by CK2, suggesting that even increased PKC binding to p53 protein cannot mediate the phosphorylation reaction.

We can conclude that cdk2/cyclin A and PKC both have a stimulatory role in the activation of sequence-specific DNA binding by latent p53, both in the oligonucleotide form and when localized within DNA molecules. The phosphorylation of p53 by CK2, which is apparently constitutively active, evokes an antagonistic effect with respect to p53 activation. In fact, Ser^{392} phosphorylation partially activates the DNA-binding function of p53, but it blocks the kinase activity of PKC, a strong p53 activator. Generally, we can say that our results support an allosteric rather than a competitive model of p53 protein latency. The allosteric model assumes that the interaction of the non-modified C-terminal domain of p53 blocks the core domain with respect to sequence-specific binding and promotes the latent state of the molecule, while modification of the C-terminus activates the binding of p53 to target DNA sequences [8,52]. Further studies should concentrate on elucidating the possible interactions of individual cell signalling pathways with regard to p53 activation and on details of the specific molecular interaction patterns.

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Binding of p53 and its core domain to supercoiled DNA

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We have compared the binding of human full-length p53 protein (p53; expressed in bacteria and insects) and its isolated core domain (p53CD, amino acids 94–312; expressed in bacteria) to negatively supercoiled (sc) DNA using gel electrophoresis and immunoblotting. Significant differences were observed; p53CD produced a relatively small and continuous retardation of scDNA, in contrast to the ladder of distinct bands formed by p53 in agarose gels. The ladder produced by full-length protein expressed in bacteria (p53b) was similar to that observed earlier with protein expressed in insect cells (p53i). Competition between scDNAs and their linearized (lin) forms showed a preference for scDNAs by both p53 and p53CD, but the ratios characterizing the distribution of the protein between sc and lin pBluescript DNAs were substantially higher for p53 (sc/lin $>$ 60 in p53b) than for p53CD (sc/lin $\approx$ 4). Strong binding of p53 to scDNA lacking the p53 consensus sequence may represent a new p53-binding mode, which we tentatively denote supercoil-selective (SCS) binding. This binding requires both the C-terminal domain and the core domain. Targets of this binding may include: (a) DNA segments defined both by the nucleotide sequence and local topology, and/or (b) strand crossings and/or bending. The binding preference of p53CD for scDNA may be due to the known nonspecific binding to internal single-stranded regions in scDNA (absent in relaxed DNA molecules) and/or to SCS binding albeit with reduced affinity due to the absence of contributions from other p53 domains.

Keywords: tumor suppressor protein p53; comparison of full-length p53 and its isolated core domain; binding of p53 to supercoiled DNA; immunoblotting of p53.

The *p53* gene, frequently called the guardian of the genome, protects vulnerable cells from malignant transformation by regulating the responses of cell growth and death to genotoxic agents (reviewed in [1–5]). The functions of this gene are closely related to the specific binding of its product, the tumor suppressor protein p53, to the DNA consensus sequence (p53CON), consisting of two copies of the sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0–13 bp [6]. The p53 protein is a metalloprotein containing one zinc atom in the core domain [7,8]. The conformation of p53 is important for the biological activity of the protein and for its binding to p53CON. It has been suggested that the dual ability of p53 to function as a tumor suppressor and to promote cell proliferation may be due to switching between wild type and mutant conformations, respectively [9–11].

Using agarose gel electrophoresis and scanning force microscopy (SFM) we have recently shown that the full-length wild-type human p53 protein binds strongly to negatively supercoiled DNA (scDNA) at native superhelix density [12]. The binding takes place both in the presence and absence of the consensus sequence (p53CON), producing several retarded bands on the gel. SFM images reveal partially or fully relaxed DNA molecules with bound p53 protein molecules. Removal of the p53 protein restores the original mobility of scDNA, suggesting that binding of the p53 protein does not change the DNA linking number. Thermally denatured plasmid DNA competed efficiently for binding of p53 to scDNA. Based on these results, we concluded that interactions of p53 with scDNA involve both the core and the C-terminal domain [12–15].

The binding of p53 to the DNA consensus sequence has been studied in detail. The cocrystal structure of residues 94–312 bound to 21 bp duplex DNA offers interesting insights into the functions of p53 [7,8]. The role of the p53CD in DNA sequence specific binding as well as its negative regulation by the C-terminus has been established [1]. The nonspecific binding to ds and ssDNAs has also been well documented. Bakalkin *et al*. [16,17] suggested that the domain responsible for nonspecific DNA binding may depend on the DNA substrate: the p53 C-terminus binds the single strand ends of DNA molecules whereas the core domain is involved in binding to internal ssDNA segments. Interactions of p53 with nucleic acid ends, Holliday junctions or irradiated DNAs involve the C-terminal nonspecific binding domain [1,4].

Compared to the well-known sequence specific and nonspecific DNA binding modes little is known about the nature of p53 binding to scDNA. Recently Mazur *et al*. [18] showed that the C-terminal domain is involved in the

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Abbreviations: p53b, full-length wild-type human tumor suppressor protein p53 expressed in bacterial cells; p53i, full-length wild-type human tumor suppressor protein p53 expressed in insect cells; linDNA, linearized DNA; p53CD, core domain (amino acids 94–312) of wild-type human tumor suppressor protein p53; p53CON, p53 consensus DNA binding sequence; scDNA, supercoiled DNA; ssDNA, single-strand DNA.

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scDNA binding. They observed no effect of a specific point mutation in the DNA binding domain (serine instead of arginine at position 249) on p53 binding to scDNA, suggesting a lack of participation of the core domain in this binding and thus another mode of nonspecific interaction by the C-terminal domain. Our studies, however, have suggested that the core domain may also be involved [12–14].

In this paper we compare the binding of p53CD and of the full-length p53 to scDNA. We show that p53CD binds preferentially to scDNA albeit with a lower relative affinity than the full-length protein, supporting our original suggestion [12] that binding of p53 to scDNA involves both the core and the C-terminal domains.

MATERIALS AND METHODS

Plasmids

Supercoiled plasmids pBluescript II SK-(Stratagene) and pPGM1 [pBluescript II SK-containing 20-mer (AGACATGCCTAGACATGCCT)] p53 consensus binding were used [12].

Protein isolation

The p53 core domain (fragment 94–312, p53CD) was expressed in bacteria, isolated and characterized as described [19]. Wild-type human full-length p53 protein was prepared and characterized as previously described [12]. Expression was in insect cells (Sf9) infected with a recombinant baculovirus and purification using a Heparin-Sepharose Hi-Trap column (Pharmacia). The same technique was used for purification of the full-length protein expressed in bacteria. The proteins migrated as single bands, visualized both by Coomassie staining and western blot analysis with monoclonal antibody DO-12 (binding to amino acids 256–270).

DNA and protein were mixed (the protein/DNA ratio is expressed in terms of p53, or p53CD, tetramers and DNA molecules) in 20 μL of the DNA binding buffer (5 mM Tris/HCl, pH 7.6, 0.5 mM EDTA, 50 mM KCl, and 0.01% Triton X-100). The samples were incubated for 30 min at 0 °C and loaded onto a 1% agarose gel containing a 0.33 × Tris/borate/EDTA buffer. Agarose gel electrophoresis was performed for 4 h at 100 V at low temperature (< 10 °C). The gels were stained with ethidium bromide and photographed. DNA or p53 bands on photographs of gels or blots were scanned and rendered digitally. The band intensities were quantified by Image QuaNT software.

Blotting and immunodetection of p53

Gels were blotted onto a nitrocellulose transfer membrane Protran R (Schleicher and Schuell, Germany) in 10 × NaCl/Cit at 5 in Hg on a vacuum blotting system (BioRad USA) [20]. The membrane was blocked with 5% nonfat milk in NaCl/P_i and the p53 proteins were detected with 1 mg·mL^{-1} primary antibody DO-11 (binding to amino acids 176–186) and/or DO-12 supernatant diluted 1 : 50–150 and secondary antibody anti-mouse IgG (peroxidase conjugate) diluted 1 : 3000. The complex was visualized with the ECL detection system (Amersham). All primary monoclonal antibodies used in this paper were kindly donated by B. Vojtesek.

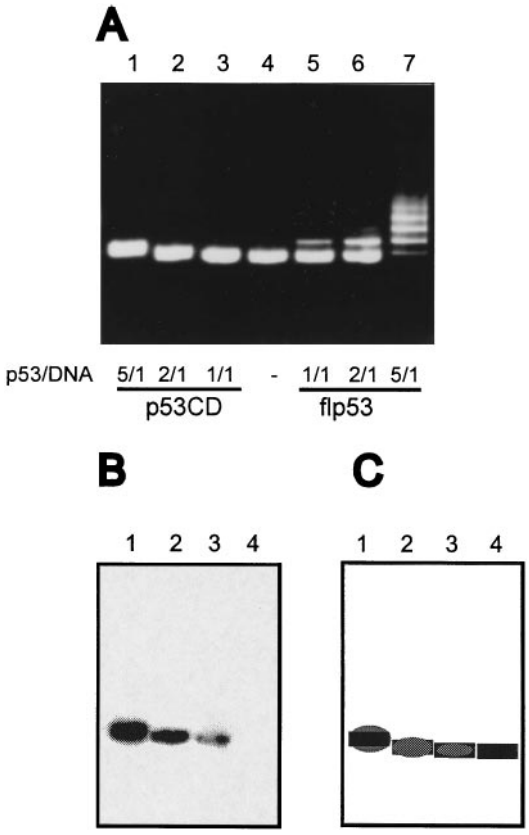

Fig. 1. Binding of p53CD and full-length p53 protein to supercoiled DNA. (A) 0.4 μg of scDNA pBluescript was incubated with core domain p53 (lanes 1–3) and full-length p53i (lanes 5–7) proteins at a molar ratios of p53 tetramer : DNA from 1 : 1 to 5 : 1. The incubation was carried out in 50 mM KCl, 5 mM Tris (pH 7.6), 0.5 mM EDTA and 0.01% Triton X-100 on ice for 30 min and stopped by adding the loading buffer. The samples were loaded on 1% agarose gel in 0.33 × Tris/borate/EDTA. DNA was stained with ethidium bromide. (B) Immunoblotting of the left half of the gel (lanes 1–4) with the monoclonal antibody DO-12. (C) Superimposition of the ethidium-stained bands (black rectangles) on agarose gel (A) and immunodetected p53CD protein (gray spots) on the blot (B).

Competition assays

Competition experiments with a mix of scDNA and *Sma*I linearized DNA (in equimolar concentrations) were performed with the total amount of DNA kept in the range of 0.8–1.6 μg per well (higher amounts of DNA and p53 were used in samples with low p53/DNA ratios to keep the p53 level detectable by monoclonal antibodies). Mixtures of linear and supercoiled forms of pPGM1 and/or of pBluescript, respectively, in a ratio 1 : 1 were incubated with p53 or p53CD (molar ratios p53 tetramer/DNA were from 1.5 to 4 : 1 and from 1 to 4 : 1, respectively) for 30 min at 0 °C. Monoclonal antibodies DO-1 (binding to amino acids 20–25) or DO-11 and DO-12 were used for immunoblotting.

For p53CD competition experiments with scDNA and heat-denatured DNA (ssDNA), 0.4 μg pPGM1 scDNA

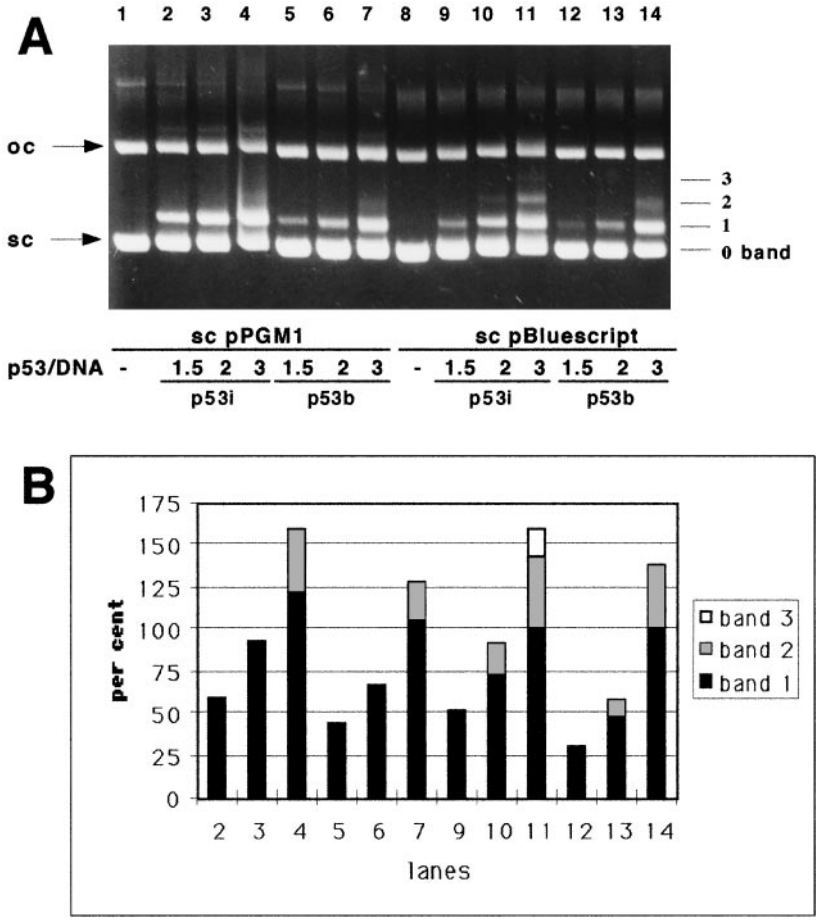

Fig. 2. Binding of full-length p53 proteins expressed in insect cells (p53i) and in bacteria (p53b) to supercoiled pBluescript and pPGM1 (containing the p53CON) DNAs at different protein/DNA ratios. (A) 0.2 μg of the DNA was incubated with p53i (for pPGM1 lanes 2–4 and for pBluescript lanes 9–11) and p53b (for pPGM1lanes 5–7 and for pBluescript lanes 12–14) at the molar ratios p53 tetramer : DNA 1.5, 2 and 3. Free scDNA band 0 and retarded bands 1, 2 and 3 are marked. (B) Bar graph of distribution of scDNA in retarded bands 1, 2 and 3 (lanes 2–14) expressed as percentage of band 1 (lane 11). Further details as in Fig. 1.

and/or pBluescript scDNA were mixed with increasing amounts of heat-denatured linear pBluescript DNA (linearized with *Sma*I), resulting in molar ratios 0.2, 0.5, 1, 2 and 5 of sc/ssDNA. The mix was incubated with p53CD (molar ratio p53 tetramer/DNA = 10 : 1) for 30 min at 0 °C. Immunoblotting of the gels was performed with a mix of monoclonal antibodies DO-11 and DO-12.

RESULTS

Core domain and full-length p53 bind to supercoiled DNA in different ways

The electrophoretic mobility of the DNA-p53 complexes depended on the p53 tetramer/DNA ratios (1–5) for the p53CD and for eukaryotic p53 protein (Fig. 1). In agreement with our previous results, p53 led to a strong retardation of the supercoiled pBluescript DNA (lacking the consensus sequence, p53CON), manifested in the form of well-resolved DNA bands, the number of which increased with increasing p53/DNA ratio (Fig. 1A, lanes 4–7). Compared to the full-length protein, p53CD under the same conditions produced a substantially smaller DNA retardation without formation of any additional resolved band (Fig. 1A, lanes 1–3). An immunoblot with the antibody DO-12 (binding to amino acids 256–270) showed increasing intensities of staining at increasing p53/DNA ratios (Fig. 1B, lanes 1–3) and a good correspondence of the ethidium-stained DNA bands with the immuno-detected protein bands (Fig. 1C).

Previous studies of p53 binding to scDNA were performed only with full-length eukaryotic p53 (expressed in insect cells) [12–15,18] active for DNA sequence-specific binding but not with p53 expressed in bacteria whose sequence-specific binding activity is latent [21]. We purified bacterially expressed p53 (p53b) and studied its binding to scDNA, and compared the binding properties of bacterially expressed p53CD with both the eukaryotic and bacterial full-length p53.

Full length eukaryotic and bacterial p53 proteins

We compared p53 expressed in insect cells (p53i, using a baculovirus expression system, which supports the majority of postranslational modifications essential for proper functioning of the eukaryotic protein), and p53b (lacking postsynthetic modification) in their abilities to bind to scDNA without (pBluescript) and with the consensus sequence (pPGM1 DNA). Figure 2 shows gel retardation of pPGM1 and pBluescript scDNAs resulting from their interactions with p53i and p53b, at three different p53/DNA ratios. For p53/DNA ratios 1.5 and 2.0 the interaction of p53i with pPGM1 DNA yielded the most intense band 1 (Fig. 2A,B, lanes 2,3). For p53/DNA 2.0 a weak retarded band 2 was observed only with pBluescript DNA. At p53/DNA 3.0 a more intense band 2 was formed both with

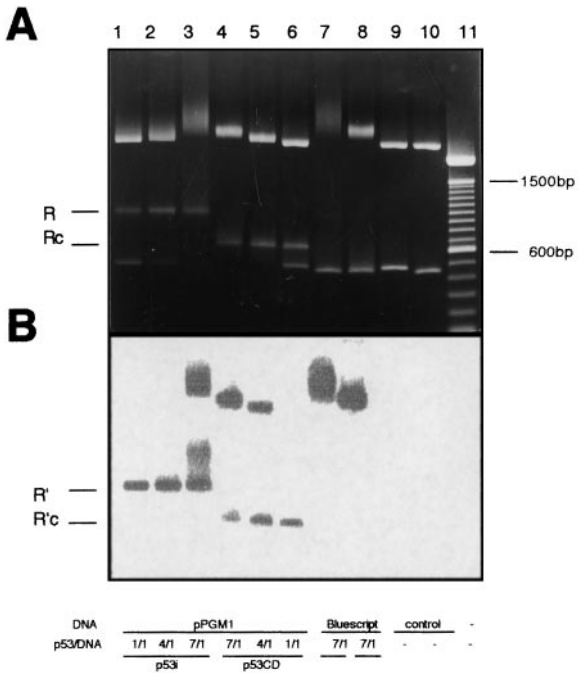

Fig. 3. Binding of p53 protein to the consensus sequence in pPGM1 DNA fragment. pPGM1 (lanes 1–6,9) was cleaved by *Pvu*II, producing 474 bp (with the p53CON) and 2513 bp (lacking the consensus sequence) fragments. pBluescript (lanes 7, 8 and 10) was digested by the same enzyme, producing 448 bp and 2513 bp fragments (both lacking the consensus sequence). (A) 0.4 μg of the DNA was incubated with p53CD (lanes 4–6) and/or fl p53 (lanes 1–3) at a molar ratios of p53 tetramer : DNA from 1.5 : 1 to 7 : 1. R and Rc, bands retarded due to the sequence-specific binding of p53i and p53CD, respectively. The pBluescript fragments were incubated at the highest p53/DNA ratios using p53i (lane 7) or p53CD (lane 8), respectively. DNA was stained with ethidium bromide. (B) Immunoblotting of the agarose gel; mix of the monoclonal antibodies DO-12 and DO-11 was used.

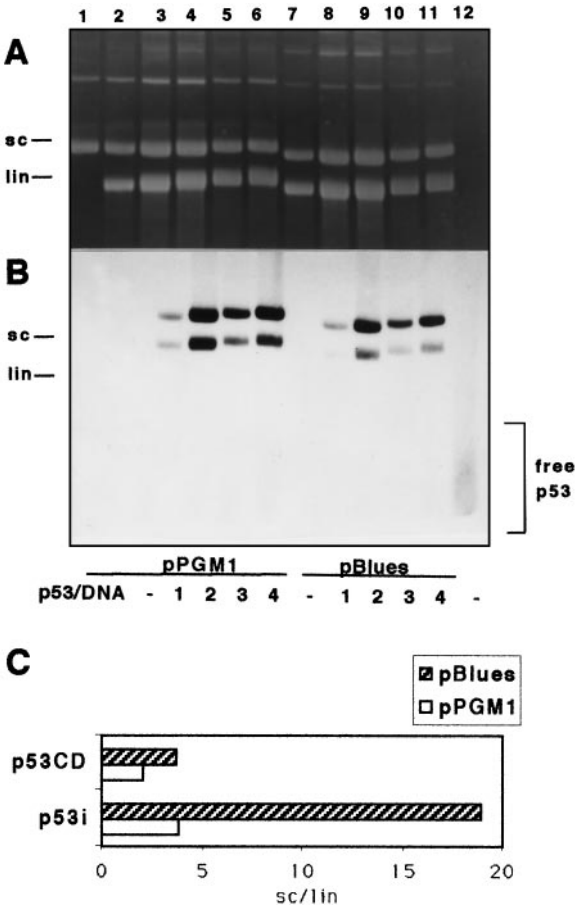

Fig. 4. Comparison of binding of p53CD to supercoiled and linear (linearized by *Sma*I) forms of pPGM1 and pBluescript plasmid. (A) 0.8 μg (lanes 2, 5, 6, 7,10 and 11) or 1.6 μg (lanes 3, 4, 8 and 9) of a mixture of linear (lin) and supercoiled (sc) forms of pPGM1 (lanes 2–6) and/or pBluescript (lanes 7–11) plasmid, respectively, in a weight ratio 1 : 1 (linear : supercoiled forms) were incubated with p53CD at molar ratios of p53 tetramer : DNA from 1 : 1 to 4 : 1 (marked below the figure). (B) Immunoblotting of the gel with the monoclonal antibody DO-12 and DO-11. Free p53 (lane 12) is marked. For results from densitometric tracing of the immunoblot see Table 1. (C) Bar graph of sc/lin ratios of band intensities obtained by densitometric tracing at protein/DNA = 1.5 for both p53i (Fig. 5) and p53CD.

pBluescript and pPGM1 DNAs (Fig. 2A,B, lanes 4,7,11,14). Under the same conditions a weak band 3 appeared with pBluescript but not with pPGM1 DNA. At least three conclusions may follow from these results: (a) both p53i and p53b generate a ladder of retarded bands on an agarose gel due to their interaction with scDNA containing or lacking p53CON; a higher number of bands was observed at higher p53/DNA with both p53i and p53b [22] (b) p53i binds to the consensus sequence in the supercoiled pPGM1 DNA which at low p53/DNA represents a preferential binding site of p53i in this DNA; (c) the presence of the consensus sequence in scDNA does not significantly influence the retarded bands induced by p53b.

The gel retardations of scDNA by p53i and p53b differed significantly from that caused by p53CD (Figs 1 and 2), suggesting that the core domain in the full-length protein cannot by itself be responsible for the ladder of bands generated by p53 on the agarose gel (Fig. 1A, lanes 4–7). The band shift caused by p53CD at p53CD/DNA of 1–5 was very small, indicating the lack of any significant relaxation of scDNA upon binding of p53CD.

Comparison of sequence-specific binding of p53CD and p53 to a DNA fragment by EMSA in agarose gel

In a previous study we used agarose gels to study the binding of p53 to both scDNA and DNA restriction fragments [12]. Here we used the same technique to compare the binding of p53CD and p53 to *Pvu*II fragments of pPGM1 (Fig. 3). p53CD led to a retarded band Rc indicative of binding to p53CON in the shorter 474 bp fragment. At p53CD/DNA = 1 a weak 474 bp band was still present, but at higher ratios this band disappeared (Fig. 3A, lanes 4–6). Band Rc was absent in the pBluescript DNA fragments (Fig. 3 A, lane 8), in agreement with the results obtained earlier with p53 [12]. Immunoblotting of the gel revealed a band Rc′ in pPGM1 (corresponding to band Rc on the gel) (Fig. 3B, lanes 4–6), which was absent with pBluescript DNA (Fig. 3B, lane 8). At p53/DNA = 1

Table 1. Competition of supercoiled and *Sma*I linearized DNAs of pPGM1 and pBluescript for p53 core domain (p53CD) or full length p53 (fl p53) insect and bacterial at different p53/DNA ratios. All data were calculated as an average made from three independent binding reactions and expressed as sc/lin ratio (for more details see Figs 4 and 5). Intensities of immunoblot staining by DO-11 and DO-12 mixture of scDNA and linDNA bands were determined by densitometric tracing for p53CD. Intensities of immunoblot staining by DO-1 of scDNA and linDNA bands were determined by densitometric tracing for insect and bacterial fl p53.

p53CD					Insect fl p53				Bacterial fl p53			
pPGM1												
p53/DNA	1/1	2/1	3/1	4/1	1.5/1	2/1	3.5/1	4/1	1.5/1	2/1	3.5/1	4/1
sc/lin (P)	1.3	1.3	2.0	1.7	3.8	2.1	1.6	1.6	1.4	4.4	2.9	1.9
pBluescript												
p53/DNA	1/1	2/1	3/1	4/1	1.5/1	2/1	3.5/1	4/1	1.5/1	2/1	3.5/1	4/1
sc/lin (B)	3.3	2.4	3.7	4.0	18.9	16.2	17.3	11.0	8.7	66.1	30.6	23.6
B/P	2.5	1.9	1.5	2.4	5.0	7.7	10.8	6.9	6.2	15.0	10.6	12

staining of the longer 2513 bp band was no longer observed (Fig. 3B, lane 6), but at higher p53/DNA ratios a significant staining of this band was detected with both pBluescript and pPGM1 DNAs (Fig. 3B, lanes 3–5,7,8).

Full-length p53i produced band R [12,23], which was much more retarded than Rc (Fig. 3A, lanes 1–3). The mobility of Rc corresponded to an apparent ≈ 700 bp DNA in contrast to the 1100 bp of band R. Differences in the mobility of the p53–DNA complexes were observed by Wang *et al*. [24] using PAGE. They showed that p53, as well as the 1–360 and 1–320 p53 fragments, led to DNA complexes migrating closely on 4% polyacrylamide gels, whereas the complex with a 80–290 fragment was much less retarded. Changes in the positions of the bands may reflect differences in the size, shape, charge and/or the oligomerization state of the p53 components in the complexes.

Competition between sc and linearized DNAs for p53CD or p53

p53 core domain. To compare the binding affinity of p53 to long linear and supercoiled DNAs equimolar concentrations of scDNA and *Sma*I linearized DNA were mixed, and p53CD at protein/DNA ratios of 1–4 was added (see Materials and methods). The ethidium-stained gel (Fig. 4A) did not show any significant differences in the retardation or intensities of the bands of sc and linDNAs. However, densitometric tracing of the p53 immunoblot (using DO-11 and DO-12 antibodies) displayed higher band intensities in scDNA (compared to the linearized forms) of both pBluescript and pPGM1 (Fig. 4B, Table 1), suggesting weak but significant preferential binding of p53CD to scDNA. The ratios of the band intensities sc/lin of pBluescript DNA were higher than in pPGM1, probably due to the presence of p53CON in lin pPGM1. These results suggest that compared to linDNA, p53CD shows a preference for scDNA both in the presence and absence of p53CON.

Full-length p53. The same experiment was also performed with p53i at protein/DNA ratios between 1.5 and 4.0. Binding of p53 to scDNAs of both pPGM1 and pBluescript resulted in a ladder of discrete bands (Fig. 5A). With increasing ratios of p53/DNA, a lowering of intensity (and final disappearance) of band 0 (scDNA only) was accompanied by formation of increasing number of retarded bands. The immunoblot of linear DNA differed from the ethidium-stained bands, in that staining of the linear pBluescript DNA was very weak (Fig. 5B, lanes 8–11), while lin pPGM1 DNA (Fig. 5B, lanes 3–6) was strongly stained. From the densitometric tracing of the immunoblot (Table 1), it is evident that the supercoiled forms of both DNAs competed strongly for p53 (Fig. 5B) in agreement with our previous results [12]. In pPGM1 the band intensity sc/lin was 3.8 at p53/DNA = 1.5; the former ratio decreased with increasing p53/DNA, suggesting a greater influence of nonspecific p53 binding at higher p53/DNA ratios (Table 1). With pBluescript at p53/DNA = 1.5 the ratio sc/lin was ≈19, i.e. almost fivefold higher than in pPGM1, implying a strong preference of p53 for scDNA as compared to the nonspecific binding to linDNA (lacking p53CON). As with pPGM1, in the case of pBluescript the sc/lin ratio decreased with increasing p53/DNA, showing a very high preference for scDNA even at p53/DNA = 4 (Table 1). Competition experiment with the full-length p53b using pBluescript DNA yielded qualitatively the same results but the ratios of sc/lin were substantially higher (Table 1), suggesting that binding of p53 to scDNA might be affected by the postranslational modification of the protein and/or that, compared to this protein, the nonspecific binding to dsDNA in p53b is decreased. Our preliminary results show substantially higher sc/lin ratios in some p53i fractions from the same preparation (differing in the extent of phosphorylation) than those presented in Table 1 (e.g. at p53/DNA 2.5, sc/lin was 109 for fraction 33 and 10 for fraction 27 which was more phosphorylated at a specific ser site than fraction 27; S. Pospisilova, M. Brazdova and B. Vojtesek, unpublished results). Detailed studies involving characterization of different fractions of p53i by monoclonal antibodies specific for different phosphorylation sites are in progress. Taken together, our results demonstrate (a) that negative supercoiling stimulates binding of both p53 and p53CD to DNA but (b) that the relative affinities for scDNA are substantially higher for full-length p53i and p53b than for p53CD (Figs 4 and 5; Table 1).

Competition between scDNA and single-stranded DNA for p53CD

p53 binds single-stranded DNA ends with the C-terminal domain and internal DNA segments via the core domain

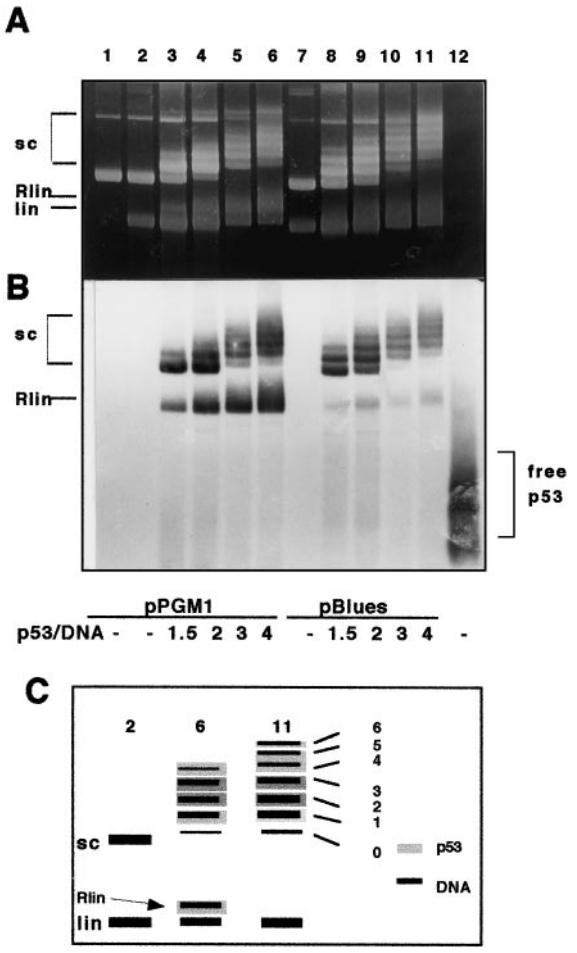

Fig. 5. Comparison of binding of p53i to supercoiled and linear (linearized by *Sma*I) forms of plasmids pPGM1 and pBluescript. (A) 0.8 μg (lanes 2, 5, 6, 7, 10 and 11) or 1.6 μg (lanes 3, 4, 8 and 9) of a mixture of linear (lin) and supercoiled (sc) forms of pPGM1 (lanes 2–6) and pBluescript (lanes 7–11) plasmid, respectively, in weight ratio 1 : 1 (linear : supercoiled forms) were incubated with p53i at molar ratios p53 tetramer : DNA from 1.5 : 1 to 4 : 1 (marked below the figure). Binding of p53 to pPGM1 linDNA produced retarded band Rlin. (B) Immunoblotting of the gel with the monoclonal antibody DO-1. Free p53 (lane 12) is marked. For results from densitometric tracing of the immunoblot, see Table 1. (C) Schematic superimposition (lanes 2, 6 and 11) of the ethidium-stained bands (black bands) on agarose gel (A) and immuno-detected p53 protein (gray strips) on the blot (B).

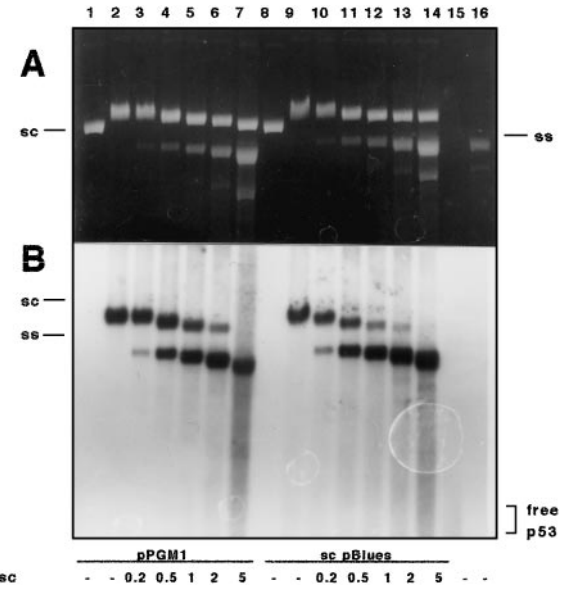

Fig. 6. Competition of scDNA and heat-denaturated *Sma*I linearized pBluescript DNA for p53CD binding. (A) 0.4 μg pPGM1 scDNA (lanes 1–7) or pBluescript scDNA (lanes 8–14) were incubated with p53CD (lanes 2–7 and 9–14) at a molar ratio of p53 tetramer : DNA = 10 : 1. Increasing amounts of heat-denaturated DNA (ss pBluescript; lanes 3–7, 10–14 and 16) was added to scDNA-p53 mixture (competitor was added in ss/scDNA molar ratios 0.2, 0.5, 1, 2 and 5 as indicated below the figure). (B) Immunoblotting of the gel with the monoclonal antibodies DO-11 and DO-12. Free p53 (lane 15) is marked. For results from densitometric tracing of the immunoblot see Table 2.

Table 2. Competition of supercoiled (sc) and thermally denatured (ss) *Sma* I linearized pBluescript DNAs for p53CD (for details see Fig. 6).

pPGM1						
ss/sc DNA	0	0.2	0.5	1.0	2.0	5.0
sc%[a]	100	90.1	58.5	39.5	21.4	0
ss%[b]	0	9.9	41.5	60.5	78.6	100
pBluescript						
ss/sc DNA	0	0.2	0.5	1.0	2.0	5.0
sc%[a]	100	79.6	38.3	17.3	6.4	0
ss%[b]	0	20.4	61.7	82.7	93.6	100

[a] Percentage of p53CD bound to scDNA as determined by densitometric tracing of DO-11 and DO-12 mixture immunoblot staining. [b] Percentage of p53CD bound to ssDNA as determined by densitometric tracing of DO-11 and DO-12 mixture immunoblot staining

[17]. We used various amounts of thermally denatured *Sma*I linearized pBluescript (ssDNA) to compete for the binding of p53CD to scDNA (see Materials and methods). From the densitometric tracing of the DO-11, DO-12 immunoblot at ssDNA/scDNA = 0.5, less than 40% of p53CD was bound to scDNA pBluescript and less than 60% to scDNA pPGM1 (Fig. 6, lanes 4 and 11; Table 2). Less then 10% of p53CD was bound to scDNA pBluescript at ssDNA/scDNA = 2 (Fig. 6, lane 13; Table 2) and at ssDNA/scDNA = 5, there was no binding of p53CD to either scDNA (Fig. 6, lanes 7 and 14; Table 2). In contrast to these results, no competition was observed in an analogous experiment using a single-stranded DNA 20-mer as a competitor at ssDNA/scDNA = 5 (data not shown). We conclude that the internal segments, but not the ends of ssDNA, are strong competitors for p53CD.

DISCUSSION

In the extensive literature on p53 interactions with DNA (reviewed in [25–27]), the effect of DNA supercoiling has received little attention. We showed recently [12] that the full-length human wild-type p53i binds preferentially to

negatively supercoiled scDNA of native superhelix density regardless of whether of the p53CON consensus sequence is present or absent. Scanning force microscopy (SFM) [12,28] (A. Schaper, D. Vik, B. Vojtesek, E. Palecek & T. M. Jovia, unpublished results) as well as electron microscopy [19] suggested that at higher p53/DNA ratios binding of both p53CD and p53i takes place at many sites of supercoiled plasmid DNA.

Role of core domain in binding of full length p53 to scDNA

In this paper we used p53CD to investigate the role of the core domain in the recognition of scDNA. There was a large difference in the binding of p53CD and full-length p53 to scDNA (Figs 1, 4 and 5, Table 1), suggesting that the strong preference of the p53 for scDNA requires the core domain as well as another region of the protein, most probably the C-terminal region [12,18]. Other recent data support an involvement of the core domain in this binding. We have found that micromolar concentrations of Zn^{2+} ions inhibit binding of p53i [13] as well as of its core domain (M. Brazdova , unpublished results) to scDNA and to p53CON in linear DNA fragments. Because the majority of putative zinc binding sites is located in the core domain, it is probable that the latter is involved not only in binding to p53CON but also to scDNA lacking p53CON. Moreover, we have found that oxidation of cysteines in p53i (which are contained only in the core domain) decreases the affinity for p53CON as well as for scDNA [14].

Recently Mazur *et al*. [18] reported a ladder of bands produced by complexes of p53i with scDNA pBluescript, confirming our earlier results [12–14]. They also showed that the C-terminal polypeptide 319–393 (peptide C) bound preferentially to scDNA pBluescript, although in contrast to p53i, the retarded DNA band was broad, as in the case of p53CD (Fig. 1) at higher p53CD/DNA ratios [29]. While the involvement of both the core and C-terminal domains appears well established [13,14,18], it is now evident that none of these domains can individually produce the same effect as the entire p53 molecule (Figs 1, 4 and 5), suggesting a complementary and/or synergistic interaction between them. It is conceivable, for example, that an important role of the core domain of the full-length protein is to achieve an optimal p53 conformation for binding to scDNA.

DNA relaxation and bending

Small global winding distortion and bending of DNA upon binding of the core domain (amino acids 96–308) were detected experimentally and predicted by molecular modeling by Nagaich *et al*. [30,31]. Recently, DNA overwinding was shown to result from p53 binding to p53CON [25]. Electron microscopic studies confirmed bending of DNA fragments upon binding of p53CD and p53i [19].

AFM images revealed an apparent partial or complete relaxation of scDNA on binding of p53 [12]. An effect which might comprise a local DNA overwinding at the single p53CON site in the pPGM1 DNA with compensatory toroidal superstructures elsewhere, in accordance with the linking number invariance [12] (A. Schaper, D. Vik, B. Vojtesek, E. Palecek & T. M. Jovia, unpublished results). However, the DNA relaxation observed in AFM images was substantially greater than in solution, as detected by electrophoresis, even assuming that the ladder of retarded bands on the gel was entirely due to such an effect. This could be the case, however, as our recent data suggested that the band ladder of p53i-DNA complexes was strongly influenced by the number of molecules and/or oligomerization state of p53i bound to the scDNA [14] (M. Brazdova, M. Fojta & J. Palecek, unpublished results).

Nature of p53 binding to scDNA

Negative supercoiling of DNA can influence p53 DNA binding in at least two ways: (a) by influencing the recognition of p53CON, and (b) in DNA lacking the consensus sequence, by configurational rearrangement of some nucleotide sequences creating new p53 binding sites. The results of Kim *et al*. [32] suggest that changes in the spatial arrangement of the p53CON sequence could modulate (enhance or reduce) p53i binding to the consensus sequence.

We have shown that both p53CD and p53i bind preferentially to scDNAs as compared to their linearized forms (Figs 4 and 5; Table 1). With p53, this preference is much stronger than with p53CD and we tentatively suggest that the strong binding of p53 to sites other than p53CON in scDNAs may reflect, at least in part, the occurrence of local supercoil-stabilized structures [33]. Such structures would reflect the influence of both nucleotide sequence and spatial disposition (influenced by supercoiling). Thus, it may not be surprising that the affinity of p53 for such sites is comparable to that for sequence-specific binding to p53CON in linear DNAs (Fig. 5; Table 1). For example, cruciforms contain four-way junctions (resembling Holliday junctions) to which p53 is known to bind preferentially [26]. Quite recently, binding of p53CD as well as of p53i to a cruciform formed by a $(AT)_{34}$ sequence in scDNA was observed by AFM [28]. The ability of p53 to recognize spatial arrangements of DNA incorporating unpaired bases such as hairpins was also demonstrated [32].

It has also been shown [18] that p53 binds preferentially to not only negatively, but also to positively supercoiled DNA, suggesting that p53 recognizes crossovers of DNA double helices, regardless of whether they arise from a linking number deficiency or excess. On the other hand, the possibility that other local structural distortions in positively scDNA are involved is not excluded. Negatively scDNA has been studied in a great detail [33] and a number of local DNA structures identified using chemical probes. Our knowledge about local structures in positively scDNA is more restricted, although information exists about increased local accessibility of bases to single-strand selective chemical probes. It was shown that an alternating adenine-thymine segment was strongly reactive to the osmium tetroxide-pyridine complex in positively supercoiled DNA [34], suggesting a local overwinding in this segment. In summary, both possibilities, i.e. p53 binding to strand crossings and to a locally deformed DNA helix in scDNAs should be considered and tested further.

Another feasible target for p53 binding which may occur both in negatively and positively scDNAs is bent DNA. The ability of both p53 and p53CD to bend DNA as a result of

sequence-specific binding was demonstrated in linear DNAs by electron microscopy [19] and other techniques [30,31,35]. Recently, Jett *et al*. [28] demonstrated the presence of bends on binding of p53CD to sc pPGM1 DNA. So far no data have been published about changes in the long DNA helix axes upon p53 binding to scDNA. This problem is currently under investigation.

We have shown that the binding of the p53 to scDNA is so strong that the protein almost fails to bind to linear pBluescript DNA in the presence of the sc form of the same DNA (Fig. 5, lanes 8–11). We conclude that at least one more mode of interaction might exist in addition to the well-known sequence-specific binding (to p53CON) and nonspecific binding to dsDNA. This new type of binding, which we tentatively call supercoil-selective (SCS) binding, is clearly observed with p53i and p53b but not with p53CD (Fig. 4, lanes 8–11). In contrast to the full-length proteins, p53CD binds significantly to lin pBluescript DNA in the presence of its sc form (Fig. 4, lanes 8–11), suggesting that the preference of p53CD for scDNA is much weaker than with p53i and p53b (Table 1). So far, strong SCS binding has only been observed with the full-length human p53 [12–15], and may play a significant biological role [12], involving, for example, stabilization of p53 (e.g. due to DNA damage) and its release in cells regulated by changes in DNA supercoiling.

The central domain of p53 has only one DNA binding site that interacts specifically with sequence motifs in dsDNA and nonspecifically with internal ssDNA regions [17]. This domain may also contain an additional nonspecific DNA binding site. Considering previous observations [17] and results contained in this paper (Fig. 6, Table 2), indicating binding of p53CD to internal segments of ssDNA but not to its ends, we conclude that the preference of p53CD for scDNA (Table 1) might be due to nonspecific binding to permanent and/or transient internal ssDNA regions known to exist in scDNA [33,36]. Alternatively, p53CD may also bind to the same sites to which full-length p53 is bound in scDNA but the affinity of p53CD is weakened due to the absence of contributions from other p53 regions.

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ORIGINAL PAPERS

Enhancement of p53 sequence-specific binding by DNA supercoiling

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Using a new competition assay, we investigated the effect of DNA negative supercoiling on the DNA sequence-specific binding (SSDB) of human wild-type (wt) p53 protein. We found that supercoiled (sc) pBluescript DNAs with different inserted p53 target sequences were stronger competitors than a mixture of scDNA pBluescript with the given 20-mer target oligodeoxynucleotide. ScDNAs were always better competitors than their linearized or relaxed forms. Two DNAs with extruded cruciforms within the target sequence were the best competitors; removal of the cruciforms resulted in a decrease of competitor strength. In contrast to the full-length wt p53, the deletion mutant p53CΔ30 and the p53 core domain (93–312 aa) showed no enhancement of p53 SSDB to scDNA, suggesting that, in addition to the p53 core domain, the C-terminal was involved in this binding. We conclude that cruciforms and DNA bends contribute to the enhancement of p53 SSDB to scDNA and that the DNA supercoiling is an important determinant in the p53 sequence-specific binding. Supercoiling may thus play a significant role in the complex p53-regulatory network.

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Keywords: competition assay; p53; DNA sequence-specific binding; supercoiled DNA; cruciform extrusion

Introduction

The p53 tumor-suppressor protein plays a critical role in the cellular response to DNA damage by regulating the expression of genes involved in controlling DNA repair, cell proliferation and apoptosis (Levine, 1997; Hupp, 1999; Jayaraman and Prives, 1999). Its mutation was found in more than 50% of human malignancies mapped predominantly to the protein core domain. This domain is responsible for sequence-specific DNA binding (SSDB) to the p53 consensus sequence (CON, two copies of the sequence 5′-RRRC(A/T)(T/A)GYYY-3′) separated by 0–13 bp (el-Deiry *et al*., 1992). It is currently believed that p53 binds optimally to this sequence as a tetramer, with each half of the p53-binding site interacting with two monomers of p53 (Arrowsmith and Morin, 1996). In addition, p53 possesses the exonuclease activity, suggesting involvement of the protein in the DNA repair (Janus *et al*., 1999a, b).

DNA topology is of fundamental importance for a wide range of biological processes including DNA transcription, replication, recombination, control of gene expression, genome organization, etc (Palecek, 1991; Bates and Maxwell, 1993; Pearson *et al*., 1996). DNA protein binding is often supercoiling dependent. The excess energy contained in supercoiled DNA (scDNA) can be relieved by protein binding. Any binding process that requires or utilizes the distortions in scDNA is favored by supercoiling. A number of DNA–protein complexes involve the wrapping of DNA around the protein, and their binding is promoted by negative supercoiling due to stabilization of writhing (Bates and Maxwell, 1993).

p53 can modulate the activity of topoisomerases either by direct molecular interactions or by transcriptional regulation, suggesting a possible relation between the p53 function and the DNA topology. Wild-type p53 protein preferentially binds to scDNA even in absence of the target sequence in the DNA molecule (Palecek *et al*., 1997, 1999; Brazda *et al*., 2000; Pospisilova *et al*., 2000; Brazdova *et al*., 2002). Strong binding of wt p53 to scDNA not containing the CON (e.g. pBluescript) was termed supercoil-selective (SCS) DNA binding. This binding was stronger in bacterially expressed p53 than in insect cell-expressed p53, suggesting the involvement of post-translational modification in this binding. The SCS/p53 binding was inhibited by some metal ions (Palecek *et al*., 1999) and by oxidation of the protein (Fojta *et al*., 1999). It was concluded that p53 binding to scDNA may involve both the core and the C-terminal domains (Palecek *et al*., 1997, 2001). By means of the gel electrophoresis and immunoblotting used in our previous experiments, it was difficult to differentiate between sequence-specific binding and SCS binding.

To overcome this difficulty, we propose a new competition assay of the effect of DNA supercoiling on p53 binding to different p53 target sequences (Figure 1). We show by this assay, which enables elimination of the effect of SCS p53 binding, that

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supercoiling enhances binding of p53 to synthetic sequences in pPGM1 and pPGM2 DNAs, as well as to *p21* and *RGC* promoters, inserted in pBluescript (Table 1). This enhancement is more intense in pPGM1, pPGM2 and *p21* than in the *RGC* insert. No such enhancement was observed with the p53 core domain (93–312 aa) and p53CΔ30 (1–363 aa), suggesting the involvement of the C-terminal domain in p53 SSDB to scDNA.

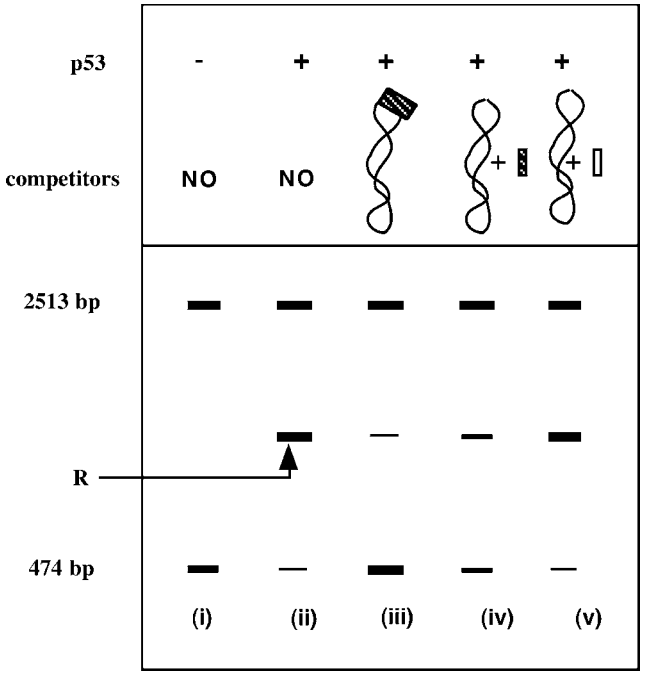

Figure 1 Diagrammatic scheme of the competition experiment, testing the ability of scDNAs to compete for sequence-specific binding of the p53 protein to (i) 474 bp *Pvu*II fragment from pPGM1 DNA. (ii) In the absence of competitors, this binding results in a well-resolved retarded band R on the agarose gel (Palecek *et al*., 1997). (iii) If p53 target (▬) containing DNA is used as a competitor, the intensity of band R strongly decreases. (iv) Substantially smaller decrease of this band is caused by a mixture of sc pBluescript DNA with a 20-mer oligodeoxynucleotide target (▪) sequence; (v) a mixture of sc pBluescript DNA with a 20-mer random sequence induces even a smaller effect on the band R intensity. Linearized and relaxed DNAs were also used in addition to the above competitors (see the text)

Results

Target sequences in scDNA are stronger competitors than the same sequences in linear DNAs (lin DNAs)

In this paper, we addressed the question whether the DNA supercoiling can enhance the p53 SSDB. To answer this question, we performed competition experiments, as described in Figure 1, using recombinant scDNAs with different p53 target sequences (Table 1). As controls, we used sc pBluescript DNA (without the target sequence) and mixtures of the latter DNA with 20-mer oligodeoxynucleotide (oligo), whose sequence corresponded to the target insert in the given scDNA (e.g. in pPGM1, Table 1). On addition of any of the competitors (in 1.5 excess to the indicator *PvuII* DNA fragments), we followed changes of indicator band R (produced by the p53–DNA complex) (Figure 2a, lanes 3–11). The intensity of this band decreased by about 80% on addition of sc pPGM1, but only by 15% on addition of pBluescript scDNAs; equimolar concentration of a nonspecific 20-mer oligo (not containing the p53 target) in sc pBluescript sample had essentially no effect (not shown). All scDNAs that contained target inserts were the strongest competitors (Figure 2b). Mixtures of pBluescript scDNA with the p53 target 20-mer oligos were the second strongest competitors. These results suggested that binding of p53 to the given p53 targets was stimulated by the DNA supercoiling. The strongest ability of scDNA to compete for p53 binding to p53 target in a lin DNA fragment was observed in pPGM1. Similar results were obtained if the radioactively labeled 20-mer oligos were used as indicators instead of the 474 bp fragment (not shown).

It has been recently reported that the affinity of p53 for targets in short oligos is lower than for those in longer DNA fragments (Espinosa and Emerson, 2001). We compared sc and *ScaI* linearized pPGM1 DNA as competitors, and found that linDNA was a much weaker competitor than scDNA (Figure 3c), differing only slightly from the mixture of lin pBluescript with the 20-mer oPGM1. Similar experiments were performed using sc and lin pP21 and pRGC DNAs (not shown). With pP21, the results were very similar to those obtained with pPGM1, but sc pRGC DNA was only a slightly better competitor than linDNA. Relaxed pPGM1 showed almost the same effect on band R as linDNA. We can thus conclude that it is not the

Table 1 p53 target sequences used in competition experiments

	Sequence[a]			
p53 target	*In oligo*		*Recombinant DNAs*	*Reference*
p21	oP21	GAACATGTCCCAACATGTTG	pPELp21 (pP21)	Nagaich *et al*. (1997)
RGC	oRGC	TGCCTTGCCTGGACTTGCCT	pPELRGC (pRGC)	Nagaich *et al*. (1997)
Synthetic1	oPGM1	AGACATGCCTAGACATGCCT	pPGM1	Palecek *et al*. (1997)
Synthetic2	oPGM2	AGACATGCCTAGGCATGTCT	pPGM2	—

[a]These sequences were either inserted in the *Hind*III site of pBluescript SK- to produce the given recombinant DNA, or used as 20-mer double-stranded ODNs in a mixture with supercoiled pBluescript DNA. For details, see Materials and methods.

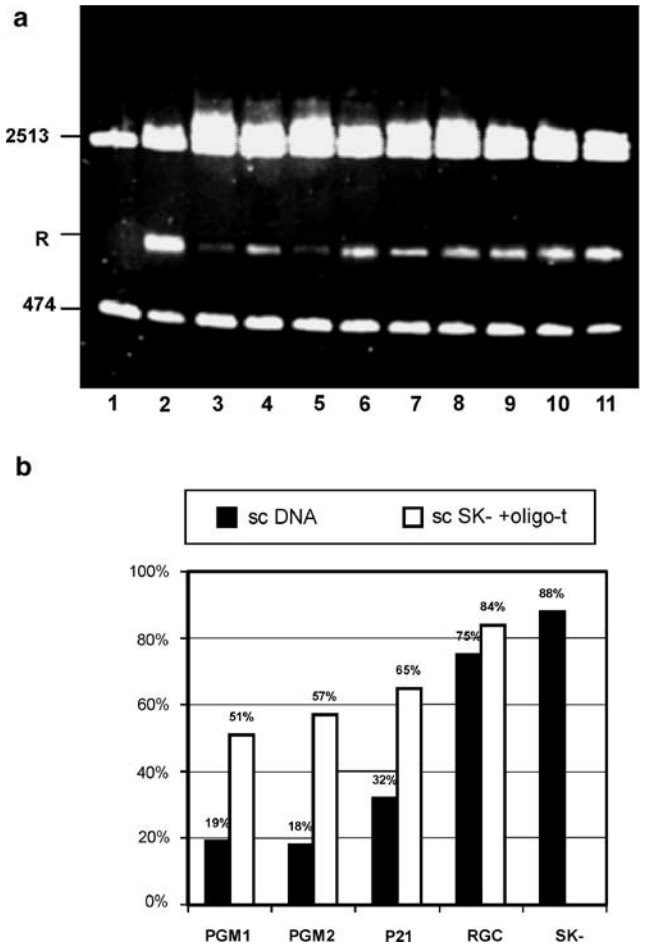

Figure 2 Competition of scDNAs (see Table 1) containing different p53 target inserts (lanes 3,5,7,9) and mixtures of pBluescript (SK-) with 20-mer oligos for p53 SSDB (lanes 4, 6, 8, 10) to p53 target in a lin 474 bp fragment. (**a**) A measure of 0.4 μg of indicator pPGM1/*Pvu*II (lanes 1–11), with 0.6 μg of competitor sc pPGM1 (lane 3), sc pPGM2 (lane 5), sc pP21 (lane 7), sc pRGC (lane 9), sc SK- (lane 11), sc SK- + oPGM1 (lane 4), sc SK- + oPGM2 (lane 6), sc SK- + oP21 (lane 8) and sc SK- + oRGC (lane 10) were incubated with 0.35 μg of wt p53 (lanes 2–11) in 50 mM KCl, 5 mM Tris-HCl (pH 7.6) and 0.01% Triton X-100 on ice for 30 min. Samples were electrophoresed on 1% agarose gel at 100 V and 4°C for 3–4 h. The molar ratio of p53 tetramer/DNA 3/1 and the change in intensity of band R (pPGM1/*PvuII*) were evaluated by densitometric tracing. (**b**) Bar graph of decreasing indicator R band intensities (lanes 3–11) expressed as the percentage of band R without competitor DNAs (lane 2). Black bars correspond to scDNAs with p53 targets (pPGM1, pP21 and pRGC) or without p53 target (pBluescript) as competitors, white bars to the mixture of sc pBluescript with 20-mer oligo-t

circularity of the DNA but its superhelicity, which is responsible for the enhancement of p53 sequence-specific binding to DNA.

p53 dissociates from linear and nicked DNAs, but not from scDNA

We were interested whether p53 already bound to the CON in a DNA fragment is dissociated from its binding site in the presence of the same sequence in scDNA. We performed the same competition experiment as in Figure 2, but first allowed the p53 protein to bind to its target in a 474 bp DNA fragment. After 30 min incubation, competitors were added in 1.5-fold excess. As competitors, we used sc pPGM1 DNA, or scDNA pBluescript with an oligo-t (oligo with p53 target sequence), or scDNA pBluescript alone. As soon as 5 min after beginning incubation with sc pPGM1 DNA, the intensity of band R decreased by 52%. Incubation with a mixture of pBluescript DNA with oligo-t resulted in a decrease of band R by 23%, while pBluescript DNA alone induced no measurable change in the intensity of band R (Figure 3b). After 60 min incubation, further decrease in the band R intensity was observed. We compared the strengths of p53 binding to the 474 bp fragment and to sc pPGM1 DNA by titrating (i) the p53–474 bp DNA complex with increasing amounts of sc pPGM1 DNA (Figure 3a, lanes 8–10) and (ii) the p53–sc pPGM1 DNA complex with increasing amounts of *Pvu*II fragments of pPGM1 (Figure 3a, lanes 3–5). Incubation with one- and twofold excesses of the sc pPGM1 DNA for 10 min strongly decreased this band intensity, and at a fourfold excess band R disappeared (Figure 3a, lanes 7–10). These results suggest that sc pPGM1 DNA is a strong competitor that induces quick dissociation of p53 from its target sequence in a lin DNA fragment. If p53 was bound first to sc pPGM1 DNA and titrated in the same way with the *Pvu*II fragments, no band R appeared even in the presence of the fourfold excess of the competitor (Figure 3a, lanes 3–5). Similarly, when p53 was first incubated with sc pP21 DNA followed by addition of a threefold excess of *Pvu*II fragments, no band R appeared, suggesting the absence of dissociation of p53 from scDNA (Figure 3c, lane 2). The same result was obtained at fivefold excess of *Pvu*II fragments (not shown). These results (Figure 3) suggest that the affinity of p53 for its target in scDNA was greater than for the target in lin DNA fragments, in agreement with the results shown in Figure 2.

We were interested whether relaxation of DNA by DN*ase*I will affect the stability of the p53–pP21 complex. We treated the scDNA–p53 complex with different concentrations of DN*ase*I for 5 min, followed by addition of a threefold excess of *Pvu*II fragments. Dissociation of p53 from the pP21 DNA increased with the DN*ase*I concentration (Figure 3c, lanes 3–5), suggesting that DNA binding of p53 to scDNA was weakened due to introduction of strand breaks and relaxation of the DNA. It is thus possible that the earlier results of the scDNA footprinting by DN*ase*I (Kim *et al*., 1999; Espinosa and Emerson, 2001) were affected by p53 dissociation from the DNA. On the other hand, inhibition of p53 SSDB to the mdmd2 promoter in scDNA was not affected by DN*ase*I cleavage (Kim *et al*., 1999), because no p53 was bound to this target.

Cruciform within the p53 target sequence in scDNA

Together with the *HindIII* site, the target sites in pPGM1 and pP_{21} formed imperfect inverted repeats with a potential ability to extrude cruciform with

single-base mismatches in each of its 13 bp stems (Figure 4a, Table 1). In addition to pPGM1, we used another DNA (pPGM2) differing in two bases and forming a perfect inverted repeat (Figure 4a). Using nuclease S1, we did not observe any indication of a cruciform presence in pP_{21} (not shown), but we detected the presence of cruciforms in sc pPGM1 and pPGM2 (Figure 4b). A comparison of the band intensities resulting from S1 cleavage suggested that the cruciform content in pPGM1 was about 40% of that present in pPGM2. On the other hand, pPGM2 was only a slightly better competitor than pPGM1 (Figure 4c, d). To understand better the effect of cruciforms on p53 binding, we compared cruciform-containing and cruciform-free pPGM2. We found that removal of the cruciform resulted in about a twofold decrease in the ability of this DNA to compete for p53 binding (Figure 4d).

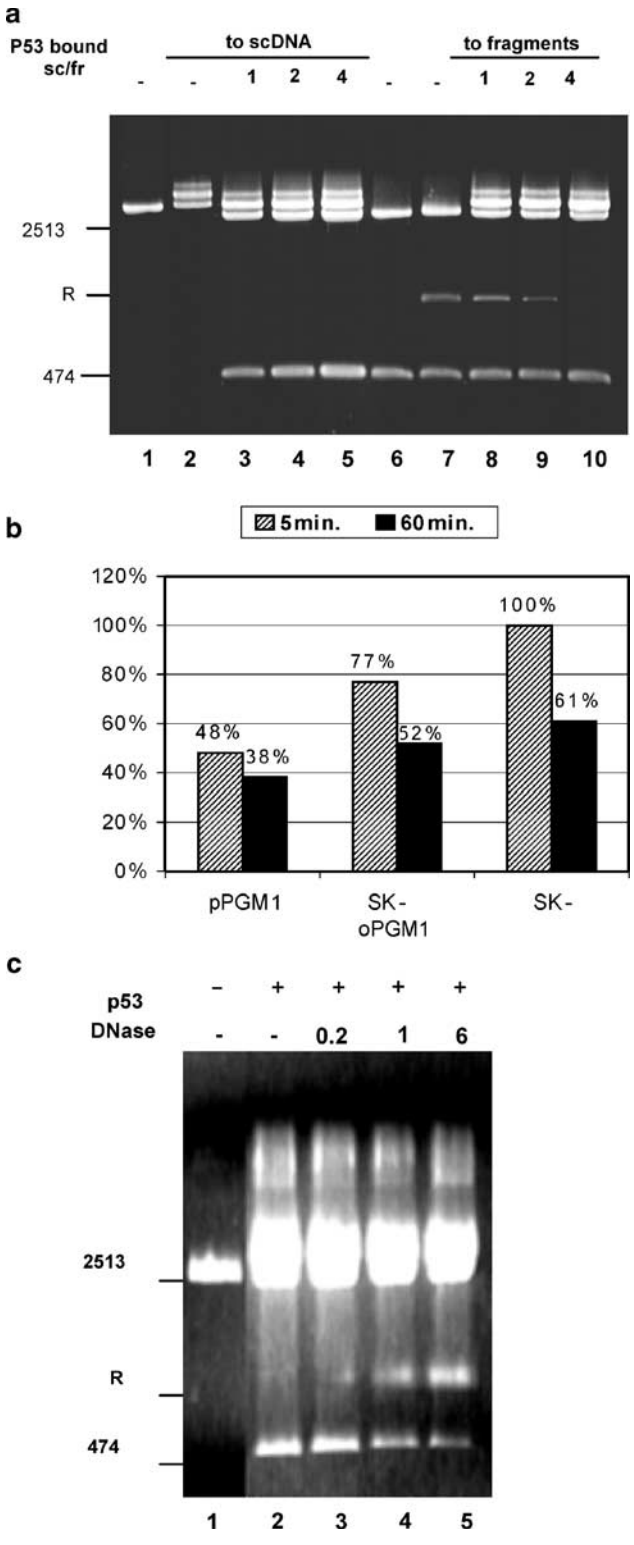

Figure 3 (**a**) scDNA-induced dissociation of p53 from its target in lin DNA. Lin DNA does not induce dissociation of p53 from sc pPGM1 (lanes 2–5). A measure of 0.4 μg of sc pPGM1 DNA (lanes 1–5) was incubated with 0.35 μg wt p53 for 15 min, followed by 15 min incubation with increasing amounts of pPGM1/*Pvu*II fragments used as competitors (0.4 μg – lane 3, 0.8 μg – lane 4, 1.2 μg – lane 5). ScDNA-induced dissociation of p53 from the p53–DNA sequence-specific complex (lanes 6–10). A measure of 0.4 μg of pPGM1/*Pvu*II fragments was incubated with 0.35 μg of wt p53 for 15 min, followed by 15 min incubation with increasing amounts of sc pPGM1 used as a competitor (0.4 μg – lane 7, 0.8 μg – lane 8, 1.2 μg – lane 9). (**b**) Bar graph showing changes in band R intensities due to incubation of the p53–linDNA sequence-specific complex with competitor scDNAs. A measure of 0.4 μg of indicator pPGM1/*Pvu*II DNA was incubated with 0.55 μg wt p53 for 30 min, followed by addition of 0.6 μg of competitors, and 5 or 60 min incubation. Sc pPGM1 DNA, a mixture of sc pBluescript SK – with oPGM1 and sc pBluescript SK – alone were used as competitors. The molar ratio of p53 tetramer/DNA was 5/1. (**c**) Dissociation of p53 from sc and nicked pP21. A measure of 0.2 pmol of sc pP21 (lanes 1–5) was incubated with wt p53 (lanes 2–4) for 10 min, followed by addition of 0 U (lane 2), 0.2 U (lane 3), 1 U (lane 4), 6 U (lane 5) of DN*ase*I in incubation buffer (6 mM $MgCl_2$, 40 mM Tris-HCl, pH 7.6). After 5 min incubation on ice, the reaction was stopped by addition of 0.5 M EDTA to 50 mM final concentration, followed by addition of 0.6 pmol of indicator pPGM1/*Pvu*II fragments (lanes 2–5) and incubation for 10 min. Other conditions are as in Figure 2

C-terminal domain is important for enhanced p53 SSDB to scDNA

We were interested whether the binding of the isolated core domain 94–312 aa (p53CD) and of the deletion mutant p53CΔ30 to the CON in scDNA will be equally enhanced as that of fl p53. We performed the competition experiments in the same way as with the fl p53 (Figure 2). We observed practically no difference in the binding affinity of the deletion mutant p53CΔ30 to lin and scDNAs (Figure 5). Similarly, p53CD displayed essentially no difference in lin and scDNAs competitor efficiencies (not shown). These results suggest that it is the C-terminal domain, which plays a critical role in the enhancement of SSDB to scDNA.

Discussion

Unconstrained supercoiling in eucaryotic cells

The effect of DNA supercoiling in regulating the p53 biological activity and the SSDB has become a topic of interest only recently (Kim *et al*., 1997; Mazur *et al*.,

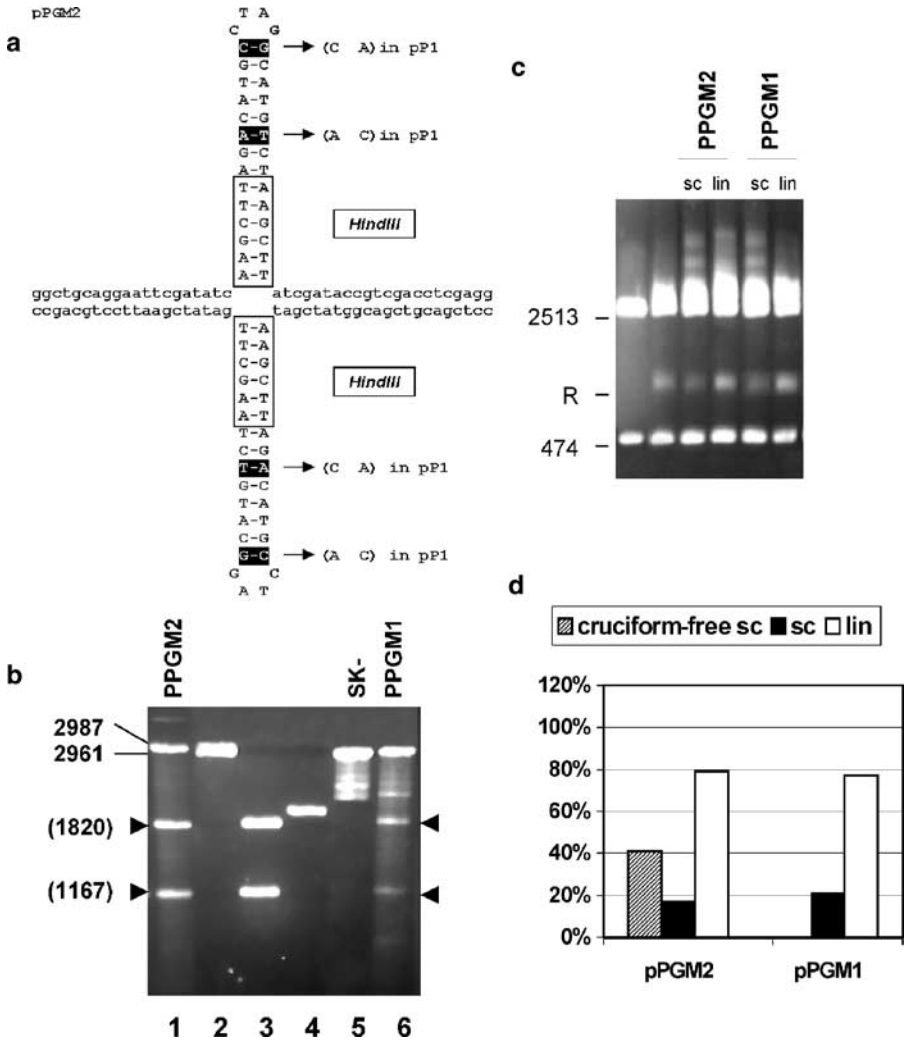

Figure 4 (**a**) Scheme of the cruciform in pPGM1 and pPGM2 DNAs. (**b**) S1 nuclease cleavage of sc pPGM1 and pPGM2 DNAs. pPGM2 (lane1), pBluescript linearized by *Hind*III restriction endonuclease (lane 2), pBluescript digested by *Hind*III and *Sca*I restriction endonucleases (used as a marker, lane 3), sc pBluescript (lane 4), pBluescript (containing no CON, serves as negative control, lane 5), pPGM1 (lane 6). Samples 1, 5 and 6 were digested by S1 nuclease followed by *Sca*I. ◀, bands resulting from the nuclease S1 cleavage at the cruciform site in pPGM2 and pPGM1 (about 1820 and 1167 bp). (**c**) Competition of sc and linearized pPGM1 and pPGM2 DNAs for p53 SSDB to a lin 474 bp fragment. A measure of 0.4 μg of indicator pPGM1/*Pvu*II (lanes 1–6), with 0.6 μg of competitor sc pPGM2 (lane 3), linearized pPGM2 (lane 4), sc pPGM1 (lane 5) and linearized pPGM1 (lane 6), was incubated with 0.35 μg wt p53 (lanes 2–6). pPGM1 and pPGM2 DNAs were linearized with *Sca*I. (**d**) The bar graph shows competitor-induced decrease of indicator R band intensities (lanes 3–6) expressed as the percentage of band R without competitor DNAs. For details, see Figure 2. Cruciform-free scDNA was a lesser competitor than cruciform-containing sc pPGM2 DNA. The presence or absence of cruciform structure in scDNAs was determined by nuclease S1 cleavage on ice (to prevent secondary cruciform extrusion at higher temperatures)

1999; Palecek *et al*., 1997, 1999; Brazda *et al*., 2000; Brazdova *et al*., 2002; Jagelska *et al*., 2002) (for a review, see Hupp, 1999). In contrast to the prokaryotic genome, the eucaryotic genome was long believed to not be under superhelical stress due to accommodation of DNA writhing around histone octamers in nucleosomes (van Holde and Zlatanova, 1994; Pearson *et al*., 1996). The actively transcribing portion of the eucaryotic genome was, however, shown to contain unconstrained supercoiling, part of which can be attributed to the process of transcription *per se* (van Holde and Zlatanova, 1994; Pearson *et al*., 1996). Using prokaryotic cells, it has been recently shown (Krasilnikov *et al*., 1999) that the effects of transcriptionally driven supercoiling are remarkably large scale *in vivo* (in the kbp range). Similarly to the transcriptional effects, intermediate supercoils are formed during DNA replication, both behind and in front of the replication fork, and superhelical stress is distributed throughout the entire replicating DNA molecule (Peter *et al*. (1998) and references therein). A number of additional processes may operate, creating transient and localized

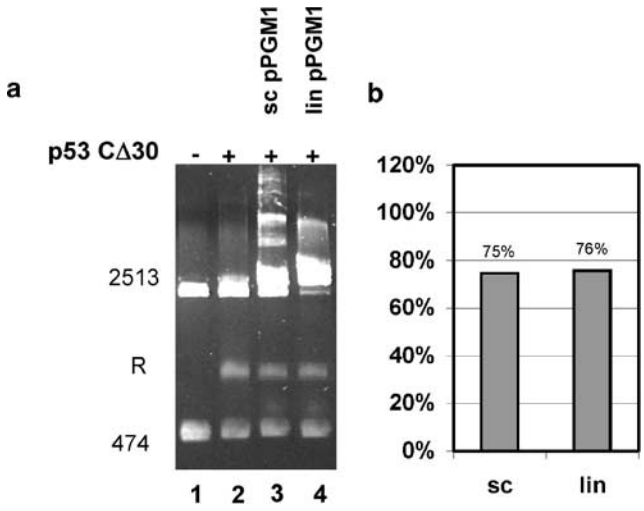

Figure 5 Effect of C-terminal deletion on p53 binding to scDNA. A measure of 0.4 μg of indicator pPGM1/*Pvu*II (lanes 2–4), with 0.6 μg of competitor sc (lane 3) or linearized pPGM1 (lane 4), was incubated with 0.27 μg of CΔ30 mutant p53 protein (lanes 2–4). (**b**) Bar graph showing changes in band R intensities C (lanes 3,4). Other details are as in Figure 2

superhelical stresses in eucaryotic DNA (van Holde and Zlatanova, 1994).

Effect of supercoiling on p53 DNA sequence-specific binding

In spite of a large amount of literature on p53 (reviewed in Levine, 1997; Jayaraman and Prives, 1999; Hupp *et al*., 2000), little is known about the mechanism by which this tumor suppressor interacts with its target genes and regulates their expression. Recently, using the natural *p21* promoter, it was reported that p53 functioned synergistically with the histone acetyltransferase p300, to activate transcription through chromatin from a distance at least 1.4 kb (Espinosa and Emerson, 2001). p53 associated with reconstituted chromatin *in vitro* at a higher affinity than with naked DNA. Higher p53 affinity for the *p21*-binding site was explained by the presence of non-B structures in the chromatin DNA. The effect of supercoiling in the plasmid was, however, not considered. Here, we show for the first time that p53 SSDB can be enhanced by negative supercoiling. The extent of the enhancement depends on the p53 target sequence (Figure 2). In pPGM1 and pPGM2, band R intensity decreased down to ~20% (Figure 4c, d), suggesting that the relative affinity of p53 to scDNA was about fivefold higher than to lin DNA, provided that in sc pPGM1 and pPGM2 the p53 protein was bound only to the CON. Assuming that p53 was distributed between the p53 target and the rest of the scDNA molecule in the same way as in the mixture of sc pBluescript DNA and oligo-t, only a 2.8-fold higher affinity for the CON in scDNA was obtained, as compared to lin DNA. In contrast to pP_{21}, pPGM1 and pPGM2, in pRGC the effect of supercoiling was very low, making it difficult to evaluate p53 affinity changes.

DNA bending and cruciform structures involving the consensus sequence can be responsible for enhanced p53 binding to scDNA

DNA bending Recently, it has been shown that HMGB1 protein can serve as an activator of p53 SSDB (Jayaraman *et al*., 1998; McKinney and Prives, 2002). HMG1-type proteins interact with DNA in a non-specific manner and, similarly to p53, they bind preferentially to scDNA (Jayaraman *et al*., 1998; Stros and Muselikova, 2000). They are able to recognize and bind a variety of local DNA structures such as DNA B–Z junctions, four-way junctions, cruciforms and stem-loops; on binding to DNA, they bend the DNA more efficiently than p53 (McKinney and Prives, 2002). It was proposed that HMG1 could help to provide the optimal DNA structure for p53 binding due to the ability of HMG1 to strongly bend the long DNA axis. It was previously shown that the SSDB of fl p53 and of p53CD induced axial bending in lin DNA (Cherny *et al*., 1999). A similar bending was observed on binding of p53CD in the waf1/cip1/p21 response element, while bending in *RGC* was much smaller (Nagaich *et al*., 1997, 1999). We have not found a cruciform structure in pP21 at native superhelix density, but the extreme flexibility/bendability of the waf1/cip1/p21 response element, observed recently by means of AFM in 168 bp DNA microcircles (Zhou *et al*., 2001), predisposes this sequence in pP_{21} to bending under superhelical stress. No such properties have been observed in the RGC sequence (Table 1) and this sequence does not seem suitable for cruciform formation. It thus appears probable that the enhanced p53 SSDB in sc pP_{21} (Figure 2) was due to a supercoil-induced bend in the p21 response element.

Cruciforms We have previously suggested that the preferential binding of p53 to scDNA may be connected with the presence of local supercoil-stabilized structures in scDNA (Palecek *et al*., 1997). It was found that the conformational state of the target sequence in oligos is an important determinant for p53 SSDB (Kim *et al*., 1997; Gohler *et al*., 2002). The enhanced p53 SSDB observed in long DNA molecules (Espinosa and Emerson, 2001) was assigned to a hypothetical structural changes in these molecules. Further, it was shown that the characteristic features of the cruciform (the single-stranded loop (Hupp, 1999), four-way junction (Lee *et al*., 1997) and the double-stranded stem) involving the target sequence, as well as the cruciform itself (Jett *et al*., 2000), represent known p53-binding sites. We detected cruciforms and observed enhanced p53 SSDB in both pPGM1 and pPGM2 scDNAs (Figure 3b, c). Removal of the cruciform in sc pPGM2 resulted in a significant decrease, but not in an elimination of the enhancement of the p53 SSDB. We may thus conclude that (i) the presence of a cruciform in the scDNA target sequence enhances p53 SSDB (Figure 4d), (ii) cruciforms are not the only source of

the enhancement of p53 SSDB to scDNA. Enhanced p53 binding was observed also with pP21, where no cruciform was detected. We found that less than half of DNA molecules with extruded cruciforms in pPGM1 was sufficient to induce approximately the same level of p53 SSDB enhancement (Figure 4d) as that produced by pPGM2 DNA containing more than a twofold higher cruciform content (Figure 4b). These results thus showed that cruciforms in pPGM1 and pPGM2 were not equal in their ability to enhance p53 SSDB. The pPGM1 cruciform containing two base mismatches in each of its stems (Figure 4a) was therefore a more efficient enhancer than the perfect cruciform of pPGM2. This is in excellent agreement with the enhanced p53 binding to mismatch-containing stem-loop oligo structures observed recently by Gohler *et al*. (2002).

Enhanced p53 SSDB to scDNA requires both the p53 core and C-terminal domains

We observed little or no enhancement in binding of p53CD or p53CΔ30 to scDNA, as compared to lin pPGM1 DNAs (Figure 5a), suggesting that the p53 C-terminus is critical for the enhanced SSDB to scDNA. It appears probable that it is the core domain that recognizes the sequence, while the C-terminus strengthens this binding by interacting with the local supercoil-stabilized structure. We have previously showed the involvement of the C-terminus in p53 binding to scDNA not containing the p53 target (Palecek *et al*., 1997; Brazdova *et al*., 2002). Here, we show that the C-terminus can also act as a positive regulator in p53 sequence-specific binding to scDNA. Positive regulation of SSDB by the C-terminus was observed by Gohler *et al*. (2002) in studies of p53 binding to stem loop oligo structures, and by McKinney and Prives (2002), who studied p53 binding to the 66 bp oligo (containing a p53 binding site) when it was locked in a microcircle. The question of the mechanism of p53 latency and the role of the C-terminus has been intensively discussed (Ayed *et al*., 2001; Yakovleva *et al*. (2001) and references therein). This discussion may soon lead to more complex models involving not only the interactions and conformations of p53, but also the global and local DNA structures, as well as the positive regulation of p53 binding. We can assume that DNA supercoiling creates a sharp bend and/or stabilizes a cruciform structure, thereby forming a DNA bend (Palecek, 1991; Yagil, 1991) involving a target sequence. It is reasonable to expect that p53 binds to these structures through both the core domain and the C-terminus. In these structures created by the DNA supercoiling, the double-stranded target sequence and segments with single-stranded character (including sharply bent DNA) can be in close proximity to each other, thus providing possibilities for simultaneous binding the C-terminus and the core of p53 (Figure 6). Such binding should be naturally stronger than binding by the p53 core domain alone to lin duplex DNAs. Earlier, we showed that in sc pPGM1 DNA, the p53 protein binds preferentially to the CON

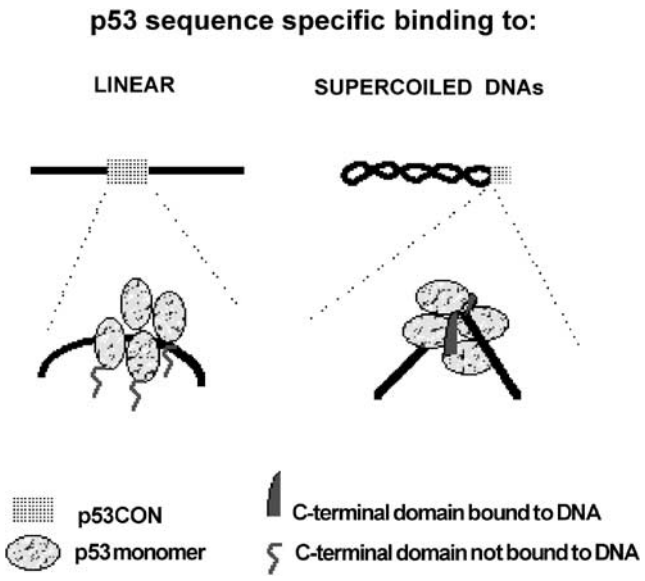

Figure 6 Scheme of the p53 SSDB to lin and scDNAs. In lin DNA, p53 binds to the CON only by the core domain producing a moderate bending of DNA (Cherny *et al*., 1999). Supercoiling can stabilize local DNA structures such as cruciforms and induce DNA bends. If the supercoil-induced DNA conformational change is sufficiently close or coincide with the target sequence, enhanced p53 binding can take place involving both the core and the C-terminal domains (see the text for details)

(Palecek *et al*., 2001); in AFM images, p53 was frequently bound to the apex of the circular pPGM1 DNA (Palecek *et al*., 1997), suggesting that p53-bound sequence specifically to the prebent DNA and/or its binding to scDNA resulted in a sharp bend in scDNA.

DNA supercoiling in the p53 regulatory network

Considering the local level of DNA supercoiling in eucaryotic DNA as an extremely dynamic system (van Holde and Zlatanova, 1994; Pearson *et al*., 1996) and the relations between the p53 and DNA topoisomerases as well as the results of this and earlier papers on p53 binding to scDNA (Palecek *et al*., 1997, 1999, 2001; Kim *et al*., 1999; Mazur *et al*., 1999; Brazda *et al*., 2000; Brazdova *et al*., 2002; Jagelska *et al*., 2002), we propose new aspects of the regulation of p53 SSDB. We predict that, in the complex p53-regulatory network, p53 DNA binding is determined not only by properties of p53 (potentially resulting from postsynthetic modification and protein–protein interactions), but also by various properties of DNA, including local DNA superhelix density and the abilities of the given p53 target and its flanking sequences to undergo conformational changes. These changes may result from various events affecting DNA supercoiling and occurring at distant DNA sites. DNA supercoiling might influence also nonspecific p53 binding and stabilization of p53 (Palecek *et al*., 1997).

It was recently reported that blocking the DNA synthesis with hydroxyurea or aphidicolin impaired p53 transcriptional activity in spite of extensive p53

post-translational modification and stabilization (Gottifredi *et al*., 2001). Under these conditions, several p53 transcriptional targets (including p21) exhibited little or no induction. To explain this finding, some factors were proposed, including the action of an as-yet-unidentified p53 specific repressor (Gottifredi *et al*., 2001), but the involvement of DNA structure at or near the p53 target has not been discussed. It has been appreciated that supercoiling may up- and downregulate some promoters, while other promoters may remain uninfluenced (reviewed in Palecek, 1991; Yagil, 1991; Pearson *et al*., 1996). Considering the recent finding that superhelical stress is distributed throughout the entire replicating DNA molecule (both *in vitro* and *in vivo*) during the DNA replication (Peter *et al*., 1998), as well as the supercoil-induced suppression (Kim *et al*., 1999) or enhancement of p53 SSDB (Figure 2), we speculate that it is the change in DNA supercoiling and concomitant changes in local DNA structures that can affect DNA transcriptional activity when DNA replication is blocked.

Materials and methods

DNA and p53 protein

Duplex oligo p21, RGC and PGM2 with *Hind*III adapters (Table 1, Figure 3a) were cut with *Hind*III and inserted into the *Hind*III site of pBluescript II SK(−). The ligated DNA was then used to transform electrocompetent cells of the Top10 strain of *Escherichia coli* (Invitrogen). DNA from single colonies selected for AMP resistance were digested with *Pvu*II and electrophoresed. Clones with a *Pvu*II fragment longer than 448 bp were subjected to amplification with M13–20 primer (5′GTAAAACGACGGCCAGT3′), and with the oligo strand of the duplex *oP21*, oRGC and/or oPGM2. The constructs with defined length (84 bp) and orientation were selected. The constructs containing the p21 target sequence were denoted as pP21 and that with the RGC as pRGC (Table 1). pPGM1 was prepared as described (Palecek *et al*., 1997). Wt human full-length (fl) p53 protein expressed in insect cells was prepared and characterized as described (Palecek *et al*., 1997). A molecular weight of 53 kDa of this post-translationally modified protein was considered in all calculations. The p53 core domain (fragment 94–312, p53CD) and p53 C-terminal deletion mutant (fragment 1–363, p53CΔ30) were expressed in bacteria, isolated and characterized as described (Brazdova *et al*., 2002).

New method for analysis of the effect of DNA supercoiling on p53 binding to different p53 target sequences

We used band R (Figure 1) as an indicator for testing the ability of scDNAs to compete for sequence-specific p53 binding. As competitors, we used (i) scDNA containing a p53 target sequence, (ii) pBluescript scDNA in a mixture with an equimolar concentration of the 20-mer oligo-t (Table 1), (iii) pBluescript scDNA in a mixture with an equimolar concentration of a 20-mer oligo, whose sequence was not a p53 target and (iv) pBluescript scDNA alone. Competitors (iii)–(iv) were used to compensate for p53 binding to other sequences in scDNA, except the p53 target. In addition, *Sca*I linearized and relaxed DNAs were used as competitors, relaxed DNAs were prepared by topoisomerase I (Promega). In the presence of a competitor, the intensity of the band R decreased. The extent of this decrease was taken as a relative measure of p53 binding to the competitor, as compared to the p53 target-containing 474 bp DNA fragment in the absence of the competitor. Standard deviations of these determinations, obtained with different competitors, were below 3.5% (calculated from 12 determinations of one competitor).

Competition experiments

In all, 0.6 μg of the competitor, 0.4 μg of the indicator pPGM1/*Pvu*II fragments and p53 protein were mixed at 1/3 molar ratio (the ratio was calculated from the concentrations of DNA and p53 tetramers) in 20 μl of DNA-binding buffer (5 mM Tris-HCl, pH 7.0, 1 mM EDTA, 50 mM KCl and 0.01% Triton X-100). The samples were incubated for 30 min on ice and loaded on a 1% agarose gel containing 0.33 × Trisborate-EDTA buffer, pH 8.0. p53 dissociation from DNA fragments or from scDNA was studied in a similar way, but the competitor was added only after the p53–DNA complex formation. Electrophoresis was performed for 4 h at 100 V (usually 4 V/cm) at low temperature (~6°C). The gels were stained with ethidium bromide, photographed, scanned and rendered digitally. The band intensities were quantified by Image QuaNT software. Electrophoresis on 4% PAGE was done in a similar way, but instead of the DNA *Pvu*II fragment, ^{32}P-labeled oPGM1 was used.

S1 nuclease cleavage

For the cruciform detection, 2 μg of plasmid was digested by S1 nuclease (2 U/ng DNA) for 20 min at 37°C in the nuclease S1 buffer (30 mM sodium acetate pH 4.6, 280 mM NaCl, 1 mM $ZnSO_4$). After the cleavage, samples were precipitated by ethanol, dissolved in the water and digested by the restriction endonuclease *Sca*I for 90 min.

Preparation of cruciform-free scDNA

Cruciform-free scDNA was prepared as described (Murchie and Lilley, 1992). Briefly, plasmid DNA in the presence of a high concentration of ethidium bromide is positively supercoiled and free of cruciforms or any local open structures. To regenerate negatively scDNA without cruciforms, we removed ethidium bromide at a low temperature in the absence of helix-destabilizing agents. We took DNA in CsCl solution, added an equal volume of 1-butanol and vortexed. After phase separation, the organic phase was removed. In order to remove CsCl, DNA was centrifuged at 14 000 g in Microcon YM-50 columns. To check the cruciform removal, the plasmid was treated with nuclease S1 on ice (to prevent cruciform extrusion at higher temperatures).

Abbreviations

scDNA, supercoiled DNA; lin, linear DNA; oligo, oligodeoxynucleotide; oligo-t, oligodeoxynucleotide with p53 target sequence; SK-, plasmid pBluescript SK-; SCS, supercoil-selective; SSDB, sequence-specific DNA binding; fl, full length; wt, wild type; CON, consensus sequence.

Acknowledgements

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Structure
Article

Interferon-Inducible Protein 16: Insight into the Interaction with Tumor Suppressor p53

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SUMMARY

IFI16 is a member of the interferon-inducible HIN-200 family of nuclear proteins. It has been implicated in transcriptional regulation by modulating protein-protein interactions with p53 tumor suppressor protein and other transcription factors. However, the mechanisms of interaction remain unknown. Here, we report the crystal structures of both HIN-A and HIN-B domains of IFI16 determined at 2.0 and 2.35 Å resolution, respectively. Each HIN domain comprises a pair of tightly packed OB-fold subdomains that appear to act as a single unit. We show that both HIN domains of IFI16 are capable of enhancing p53-DNA complex formation and transcriptional activation via distinctive means. HIN-A domain binds to the basic C terminus of p53, whereas the HIN-B domain binds to the core DNA-binding region of p53. Both interactions are compatible with the DNA-bound state of p53 and together contribute to the effect of full-length IFI16 on p53-DNA complex formation and transcriptional activation.

INTRODUCTION

Interferons (IFNs) are cytokines involved in diverse biological functions including regulation of antiviral, antibacterial, immune, and inflammatory responses (Gutterman, 1994; Stark et al., 1998). The effects of IFN stimulation are often manifested through activation of IFN-inducible genes. Although many genes are known to be activated by this pathway, their biological and functional importance remains unclear.

The IFN-inducible HIN-200 gene family encodes a class of homologous proteins that share a 200-amino acid signature motif (HIN) (Dawson and Trapani, 1996). Four human (IFI16, MNDA, AIM2, and IFIX) and five mouse (p202a, p202b, p203, p204, and p205) members of this family have been identified (Figure 1A). Most of the HIN-200 proteins possess two major protein domains. At the N terminus, there is a conserved α-helical PYRIN domain, which belongs to the death domain-containing protein superfamily involved in apoptosis, inflammation, and immune responses (Hiller et al., 2003; Stehlik and Reed, 2004). At the C terminus, all HIN-200 proteins possess either one or two copies of a conserved HIN domain, which has been implicated in DNA binding, as well as in mediating protein-protein interactions for transcriptional regulation (Koul et al., 1998; Xin et al., 2003; Ludlow et al., 2005; Yan et al., 2008).

The mouse protein p202a, containing two HIN domains exclusively, is the most studied member of the HIN-200 family. The HIN repeats in p202a have been shown to interact with numerous transcription factors including pRB, p53, NFκB, AP-1, MyoD, and E2F, indicating that the HIN domain may serve as a scaffold to assemble large protein complexes to modulate the transcription of target genes (Choubey, 2000; Xin et al., 2003). The human HIN-200 homolog IFI16, whose primary sequence predicts a PYRIN domain and two tandem HIN domains, has also been implicated in binding to pRB, E2F1, p53, and BRCA1 (Johnstone et al., 2000; Aglipay et al., 2003; Xin et al., 2003). Recently, human AIM2 was shown to be essential for sensing cytoplasmic foreign DNA through its HIN domain and interacting with an apoptosis-associated protein, ASC, via its PYRIN domain, leading to activation of inflammatory responses (Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009).

The observation that these HIN-200 proteins interact with several cellular regulators involved in cell cycle control, proliferation, differentiation, and apoptosis hints that the physiological role of HIN-200 proteins may lie beyond the IFN system (Ludlow et al., 2005; Ding et al., 2006). Indeed, IFI16 is widely expressed in normal human endothelial and epithelial cells in addition to hematopoietic cells (Gariglio et al., 2002; Wei et al., 2003; Raffaella et al., 2004; Ludlow et al., 2005). Several studies have demonstrated that loss or reduced expression of IFI16 is often associated with various forms of human cancers, including those of the pancreas, prostate, and breast (Trapani et al., 1992; Xin et al., 2003; Fujiuchi et al., 2004); leading to the notion that IFI16 may play an important role in tumor suppression. However, the molecular mechanism by which IFI16 exerts its activity

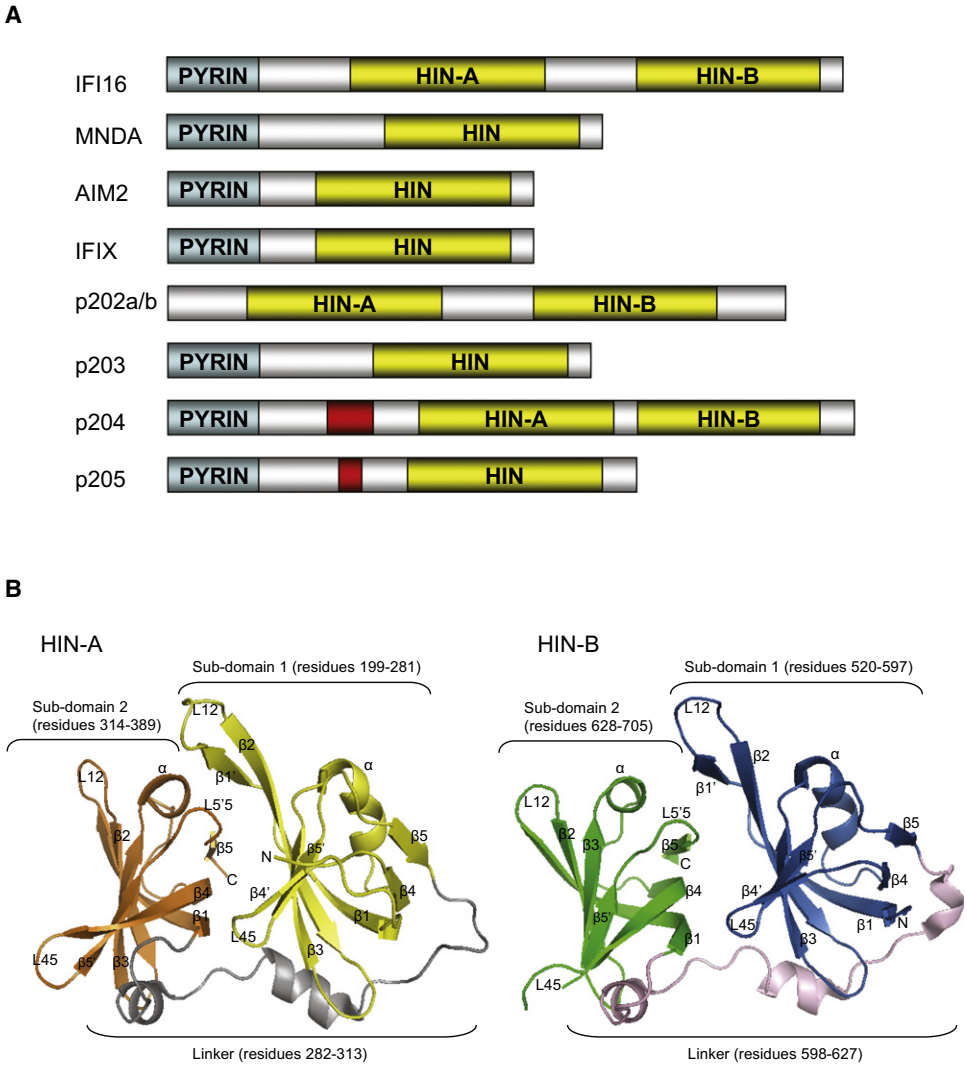

Figure 1. The HIN Domains of IFI16

(A) Overall domain architecture of the HIN-200 proteins in human (IFI16, MNDA, AIM2, IFIX) and mouse (p202a/b, p203, p204, p205). The PYRIN and HIN domains are indicated by the cyan and yellow shadings, respectively. Red shadings highlight S/T- rich region.

(B) Structure of HIN-A (left) or HIN-B (right) domain of IFI16 in cartoon representation. N and C termini are indicated.

See also Figure S1.

remains unknown. Understanding the IFI16-mediated protein-protein interaction will provide insight into its mechanism of action and function.

Inactivation of the p53 tumor suppressor gene occurs in more than half of human cancers, and loss of p53 function is a fundamental step in the pathogenesis of most human cancers (Vogelstein and Kinzler, 1992). The p53 protein functions as a transcription factor and can transactivate cellular genes through sequence-specific DNA binding to their promoters (Prives and Manley, 2001). Although the physiological significance of IFI16 in p53 biology is still under investigation, it has been reported to regulate p53-mediated transcriptional activation (Johnstone et al., 2000; Fujiuchi et al., 2004; Xin et al., 2004). In addition, IFI16 also plays a role in p53-mediated transmission of apoptosis signaling in response to DNA damage, by upregulating p53 and downregulating MDM2 (Fujiuchi et al., 2004). Furthermore, tetracycline-regulated IFI16 also induced apoptosis in the presence of ionizing radiation when coexpressed with p53 in human bladder carcinoma p53-deficient EJ cells (Fujiuchi et al., 2004). Together, these studies suggest that IFI16 is involved in the regulation of p53 stability, p53-mediated transcriptional activation, and apoptosis.

In order to better understand the molecular details and mechanism of p53 regulation by IFI16, here, we present the crystal structures of the HIN-A and HIN-B domains of IFI16 determined at 2.0 and 2.35 Å resolution, respectively. These structures represent the first experimentally determined HIN domain structures reported in the HIN-200 protein family. Although they are structurally similar, HIN-A recognizes the C terminus of p53, whereas HIN-B binds to the DNA-binding region of p53,

highlighting important functional differences between the two HIN domains. Furthermore, the two HIN domains of IFI16 may work cooperatively to stimulate the transcriptional activity of p53. Together, our structures provide a molecular description of the conserved HIN domain in this family, and offer insight into the mechanism by which IFI16 recognizes, binds, and potentially regulates p53.

RESULTS AND DISCUSSION

Overall Structures of IFI16 HIN-A and HIN-B Domains

The crystallized proteins of the first (HIN-A) and second (HIN-B) IFI16 HIN domains span residues 192–393 and 515–710, respectively. Data collection and refinement statistics are summarized in Table 1. As shown in Figure 1B, each HIN domain adopts an overall α/β fold that is further organized as two subdomains oriented in tandem. The first subdomain (residues 199–281 in HIN-A; residues 520–597 in HIN-B) is composed of eight β strands and one α helix, forming a globular barrel with the approximate dimensions 13 × 18 × 36 $Å^3$ for HIN-A and 14 × 18 × 35 $Å^3$ for HIN-B. Correspondingly, the second subdomain (residues 314–389 in HIN-A; residues 628–705 in HIN-B) consists of six β strands and one α helix, forming a second globular barrel with the approximate dimensions 11 × 19 × 21 $Å^3$ for HIN-A and 10 × 20 × 22 $Å^3$ for HIN-B. In both cases the two subdomains are connected by an extended interdomain linker (residues 282–313 in HIN-A; residues 598–627 in HIN-B) with two α helices in between for HIN-A, and three α helices for HIN-B.

Structural comparison of the individual IFI16 HIN-A or HIN-B subdomain with known folds using the DALI algorithm (Holm and Sander, 1993) revealed that each subdomain of HIN-A and HIN-B has high homology to the oligonucleotide/oligosaccharide binding (OB) fold found in many proteins (see Figure S1A available online), including replication protein A (RPA) (Bochkarev et al., 1997; Deng et al., 2007), single-stranded DNA-binding protein (SSB) (Kerr et al., 2003), aspartyl-tRNA-synthetase (AspRS) (Charron et al., 2003; Poterszman et al., 1994), BRCA2 (Yang et al., 2002), and telomere-binding protein α subunit (α-TEBP) (Classen et al., 2001; Theobald and Schultz, 2003; Buczek and Horvath, 2006), with Z-scores ranging from 7.1 to 11.3. This finding is consistent with prediction based on comparative sequence analysis that HIN domain contains two OB folds (Albrecht et al., 2005). A canonical OB fold consists of two three-stranded antiparallel β sheets packed orthogonally, forming a closed β-barrel in a 1-2-3-5-4-1 topology, in which the β1 strand is shared by both sheets (Theobald et al., 2003). Comparison of the IFI16 HIN subdomains with these well-characterized OB folds reveals significant variability in the length and arrangement of the β strands in forming the barrel, as well as the L12 and L45 loops, which are known to be important for recognizing and binding to oligonucleotide or peptide substrates (Theobald et al., 2003). For example the L12 and L45 loops of each HIN subdomain consist of less than five residues, whereas for other proteins these corresponding loops, especially L45, can be greater than 15 residues (e.g., AspRS, RPA70, α-TEBP). In addition, helical turn variations in the α helix connecting strands β3 and β4 are also observed among the HIN subdomains and other OB-fold proteins. This α helix is present in all four HIN subdomains and is another conserved feature of the OB-fold template. This helix packs against the open edge of the barrel and may be essential for structural integrity of the fold (Theobald et al., 2003; Kerr et al., 2003). In the case of SSB and α-TEBP, the OB fold of these two proteins uses a distorted helical turn to mimic the α helix and, thus, accomplish the same effect (Kerr et al., 2003). In total, over 50 structural homologs of IFI16 HIN subdomains were identified, reflecting the widespread occurrence of the OB fold. Surprisingly, when a DALI search was performed using the complete HIN-A or HIN-B domain (rather than the individual subdomain), the closest match was the structure of the DNA-bound form of RPA 70 kDa subunit (RPA70, PDB: 1JMC) (Bochkarev et al., 1997), which contains two consecutive OB folds but is only weakly superimposable with root-mean-square deviation (rmsd) of 5.8 Å (over 173 C_α atoms) and 5.6 Å (over 170 C_α atoms), respectively (Figure S1B). Such a large deviation is primarily seen in the size and conformation of various secondary structural elements and variable loops, as well as in the interdomain linker, which could possibly influence the subdomain/subdomain orientation. In fact the interdomain linker bridging the two OB folds of RPA70 is highly flexible and can adopt different conformations in the absence of DNA. Moreover, unlike the HIN domain of IFI16, the two OB folds of RPA70 do not come close enough to interact with each other without DNA (Bochkareva et al., 2001).

Despite a moderate sequence identity of ~40% between the HIN-A and HIN-B domains, their crystal structures can be superimposed readily with rmsd of 1.3 Å for all the main chain Cα atoms (Figure S1C). However, differences between the HIN-A

Table 1. Summary of Data Collection and Refinement Statistics

	HIN-A (Se-SAD)	HIN-B (Native)
Data Collection		
Space group	$P2_1$	$P2_12_12_1$
Unit cell parameters		
a, *b*, *c* (Å)	43.3, 88.9, 112.8	43.0, 92.9, 100.3
α, β, γ (°)	90.0, 99.4, 90.0	90.0, 90.0, 90.0
Wavelength λ (Å)	0.97918	1.0
R_{merge} (%)	7.2 (38.9)	6.6 (37.9)
I/σ(I)	31.4 (2.7)	26.4 (4.8)
Completeness (%)	96.4 (85.7)	99.9 (100)
Redundancy	4.1 (3.6)	7.2 (7.3)
Refinement		
Resolution (Å)	41.3–2.0 (2.07–2.00)	42.1–2.35 (2.43–2.35)
Number of unique reflections	55105	17421
R_{work}/R_{free} (%)	20.8/26.0	22.9/29.2
Number of atoms		
Protein	6160	2938
Water	159	17
Mean B factor ($Å^2$)	47.7	55.6
Rms deviation from ideal geometry		
Bond length (Å)	0.011	0.016
Bond angle (°)	1.332	1.466
PDB accession code	2OQ0	3B6Y

Values in parentheses are for the highest resolution shell.

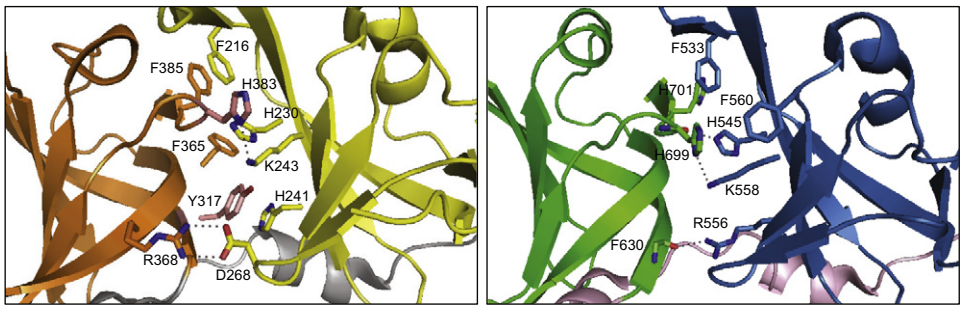

Figure 2. Subdomain/Subdomain Interactions of IFI16 HIN Domains
Close-up view of residues involved in subdomain/subdomain interaction for IFI16 HIN-A (left) and HIN-B (right).

and HIN-B domains are seen in various loop regions and the orientation of nearby secondary structural elements. For example the L12 loops in subdomain 1 and subdomain 2 have maximal shifts of 8.1 and 3.1 Å, respectively, when HIN-A and HIN-B are superimposed. Other substantial differences between HIN-A and HIN-B are observed in the conformations of the L45 and L3α loops in subdomain 2, with deviations of 2.0 and 2.9 Å, respectively.

Subdomain/Subdomain Interactions of IFI16 HIN Domains

For each HIN domain of IFI16, there appears to be extensive interactions at the interface between its two OB-fold subdomains. First, recombinant proteins comprising only one of the subdomains were insoluble (data not shown), suggesting that both subdomains may be required to provide proper structural stability for the HIN domain. Second, several key intramolecular interactions are evident between residues from each subdomain (Figure 2). The observation that various aromatic interactions, in addition to other noncovalent contacts, are present at the subdomain/subdomain interface of each HIN domain may explain why disrupting these interactions was detrimental to the overall fold of the structure. Furthermore, these aromatic residues are mostly conserved across the HIN-200 protein family, further suggesting that they are likely the key components of the HIN domain.

To further evaluate if the interface formed by the OB-fold subdomains is interaction specific and not an artifact of crystal packing, the Protein Interfaces, Surfaces and Assemblies service PISA at European Bioinformatics Institute (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) was utilized. As presented in Table 2, various interaction and thermodynamic parameters associated with interface analysis were acquired for HIN-A and HIN-B, and in comparison with that of other consecutive OB fold-containing proteins RPA70 (DNA bound; PDB: 1JMC; or unbound, PDB: 1FGU), α-TEBP (PDB: 2I0Q), and BRCA2 (PDB: 1MIU). The Δ^iG P value is a measure of interface specificity. It indicates the probability of getting a smaller than obtained solvation free-energy gain Δ^iG upon interface formation, if interface atoms were selected randomly from a protein surface. A P value of 0.5 implies that the formed interface is not unique, whereas P value >0.5 indicates that the interface is less hydrophobic than it could be and is likely to be an artifact of crystal packing. Finally, P value <0.5 signifies that the interface formed has surprising hydrophobicity, implying it is interaction specific (Krissinel and Henrick, 2007). Comparing these Δ^iG P values, we can easily observe that the interface formed by the tandem OB-fold subdomains of HIN-A (P value 0.179) or HIN-B (P value 0.232) is the most interaction specific. Interestingly, unlike HIN-A or HIN-B, the interface formed by the OB-fold subdomains of RPA70 or α-TEBP appears likely to be the result of crystal packing because the Δ^iG P value for each was above 0.5. Taken together, this suggests that the HIN domains of IFI16 may constitute a unique arrangement of closely interacting tandem OB folds that are stabilized by an extensive array of hydrogen bonding and aromatic interactions.

p53 C Terminus Binds to IFI16 HIN-A Domain

The role of IFI16 in p53-mediated transcriptional activation and apoptosis was first suggested based on its physical association with p53. Johnstone et al. (2000) mapped the interaction of the C terminus of p53 to a large region of IFI16 encompassing the first HIN-A domain (residues 155–476), but not the region containing the second HIN-B domain (residues 477–729). To evaluate whether the HIN-A domain itself is sufficient for interacting with p53, we performed a protein-protein interaction assay using recombinant His-tagged IFI16 HIN-A domain (residues 192–393) with GST-p53 C terminus (residues 355–393), GST-p53 C terminus with the tetramerization domain (residues 311–393), or GST alone. A similar assay was also conducted using His-tagged IFI16 HIN-B domain (residues 515–710). As shown in Figure 3A, the HIN-A domain was able to bind both constructs of p53 C terminus, indicating that residues outside of the structured HIN-A domain are not required for p53 interaction. Secondary structural analyses on these regions (residues 155–191 and 394–476) indicate that they are primarily unstructured (data not shown) and, thus, are not likely to participate in binding p53 C terminus, which also lacks secondary structural features. Despite having a similar fold to HIN-A, the HIN-B domain was not able to bind any of the p53 C terminus constructs.

To determine the strength of the interaction, we next measured quenching of the intrinsic tyrosine fluorescence of HIN-A or HIN-B domain upon addition of the p53 C terminus (residues 355–393), which does not possess any tyrosine residue. HIN-A domain contains five tyrosine residues (Y218,

Table 2. Summary of PISA Analysis of OB-Fold Subdomain/Subdomain Interaction

	Subdomain 1		Subdomain 2		Interface	ΔiG	ΔiG	N_{HB}	N_{SB}	N_{DS}
	N_{at}	N_{res}	N_{at}	N_{res}	area (Å^2)	kcal/mol	P Value			
HIN-A: 2OQ0	65	20	53	11	567.4	−8.8	0.179	4	4	0
HIN-B: 3B6Y	49	15	45	11	484.4	−5.2	0.232	3	0	0
RPA70A: 1JMC	34	12	25	11	278.9	0.1	0.650	5	2	0
RPA70A: 1FGU	6	1	7	2	52.5	−0.2	0.576	0	0	0
TEBP: 2I0Q	85	19	75	17	689.0	−4.5	0.556	12	5	0
BRCA2: 1MIU	48	14	37	10	394.1	−4.5	0.415	3	1	0

N_{at}, number of interfacing atoms in the corresponding subdomain; N_{res}, number of interfacing residues in the corresponding subdomain; N_{HB}, number of hydrogen bonds across the interface; N_{SB}, number of salt bridges across the interface; N_{DS}, number of disulfide bonds across the interface.

Y246, Y267, Y317, Y324), two of which (Y218, Y267) are surface exposed. Similarly, HIN-B domain contains five tyrosine residues (Y535, Y579, Y589, Y648, Y649) with three being surface exposed (Y535, Y589, Y649). Two of the three surface-exposed tyrosine residues of HIN-B (Y535, Y589) are situated near the same region on the first OB-fold subdomain as that of HIN-A. As demonstrated by the titration curve (Figure 3B), p53 C terminus binds IFI16 HIN-A domain with an apparent affinity of K_d ~20 μM. However, the same fragment of p53 C terminus did not show saturated binding to the HIN-B domain (K_d > 200 μM). This result further supports our findings that HIN-A is the binding site for the C terminus of p53, suggesting that distinct features present in HIN-A may be essential for p53 recognition.

IFI16 HIN-A Domain Enhances p53-DNA Complex Formation

The C terminus of p53 (residues 355–393) has been implicated in several important activities of p53, including autoregulation, protein-protein interactions, and nonspecific DNA binding (Hupp et al., 1992; Gu and Roeder, 1997; Anderson et al., 1997; Brazda et al., 2000; Ayed et al., 2001; Liu et al., 2003; Weinberg et al., 2004). It has been suggested that the binding of IFI16 to p53 C terminus may be important for p53 binding to DNA. This was initially shown by polyacrylamide electrophoretic mobility shift assays (EMSAs) in which the nuclear lysates of Mol-4 T cells, containing constitutively expressed IFI16 and p53, could bind p53-consensus oligonucleotides, and that binding was reduced in the presence of an IFI16 antibody (Johnstone et al., 2000). However, it was not clear whether IFI16 was associating with p53 directly.

Here, we performed EMSA using purified recombinant proteins of IFI16 HIN-A domain (residues 192–393) and p53 (residues 82–393) to assess whether the direct interaction between the two proteins can influence p53's sequence-specific DNA-binding function. This p53 variant contains the central core sequence-specific DNA-binding domain, the tetramerization domain, and the C-terminal regulatory domain. As shown in Figure 4A, addition of IFI16 HIN-A protein was able to augment p53 (residues 82–393)'s binding to its consensus double-stranded DNA sequence 5′-GGACATGCCCGGGCATGTCC-3′, forming a stable protein-DNA complex in a dose-dependent manner (compare lane 6: 0 μM IFI16 to lane 10: 80 μM IFI16). Increasing amounts of IFI16 HIN-A protein by itself on the other hand, did not show any binding to the same DNA molecules (lanes 1–5).

We also performed EMSA using agarose gel to determine the effect of IFI16 HIN-A domain on p53 (residues 82–393) binding to larger DNA molecules (474 base pairs [bp]) containing p53-consensus sequence (Figure 4B). In the absence of HIN-A, binding of p53 to its DNA target was not evident at low concentration (lane 2). However, in the presence of increasing amounts of HIN-A, p53 was able to bind to the probe, as indicated by the formation of retarded bands with corresponding disappearance of free DNA (lanes 3–5). Notably, a more slowly migrating band was also observed in lanes 4 and 5, indicative of higher molecular weight protein-DNA complexes. Using agarose EMSA, we also showed that IFI16 HIN-B domain, which does not interact with p53 C terminus, was not capable of activating p53 (residues 82–393)'s DNA-binding activity (Figure 4B, lanes 6–9). These results are consistent with previously reported data in which proteins that bind to the C terminus of p53 have been shown to promote stable formation of sequence-specific p53-consensus DNA complexes in EMSA experiments (Hupp et al., 1995; Anderson et al., 1997; Brazda et al., 2000; Sarkari et al., 2010). Thus, IFI16 shows biochemical features of other C-terminal p53-binding proteins and can influence p53's DNA-binding properties.

p53-Binding Surface of IFI16 HIN-A Domain

Given the overall structural similarities of HIN-A and HIN-B domains of IFI16, we investigated the structural features that are unique to HIN-A domain and mediate interaction with p53 C terminus. Comparing the electrostatic potential surfaces of the two HIN domains based on our crystal structures (Figure 5A), we hypothesized that the interaction with the basic C terminus of p53 may be mediated by an overt acidic/hydrophobic patch observed on the surface of HIN-A (formed by residues Y218, T220, E222, Y267, E272, E381), but not HIN-B domain of IFI16. This surface encompasses the two exposed tyrosine residues that presumably gave rise to the changes in fluorescence signal upon p53 binding (Figure 3B) and is in an area of the OB fold commonly found to interact with binding partners (Theobald et al., 2003). To further elucidate the molecular basis of p53 binding to HIN-A, we performed mutagenesis studies examining the roles of the aforementioned surface-exposed residues in mediating p53 interaction and activation (Figure 5B). Compared to activation of p53 (residues 82–393) by wild-type HIN-A domain (lane 3), T220A, E381A, Y218A,

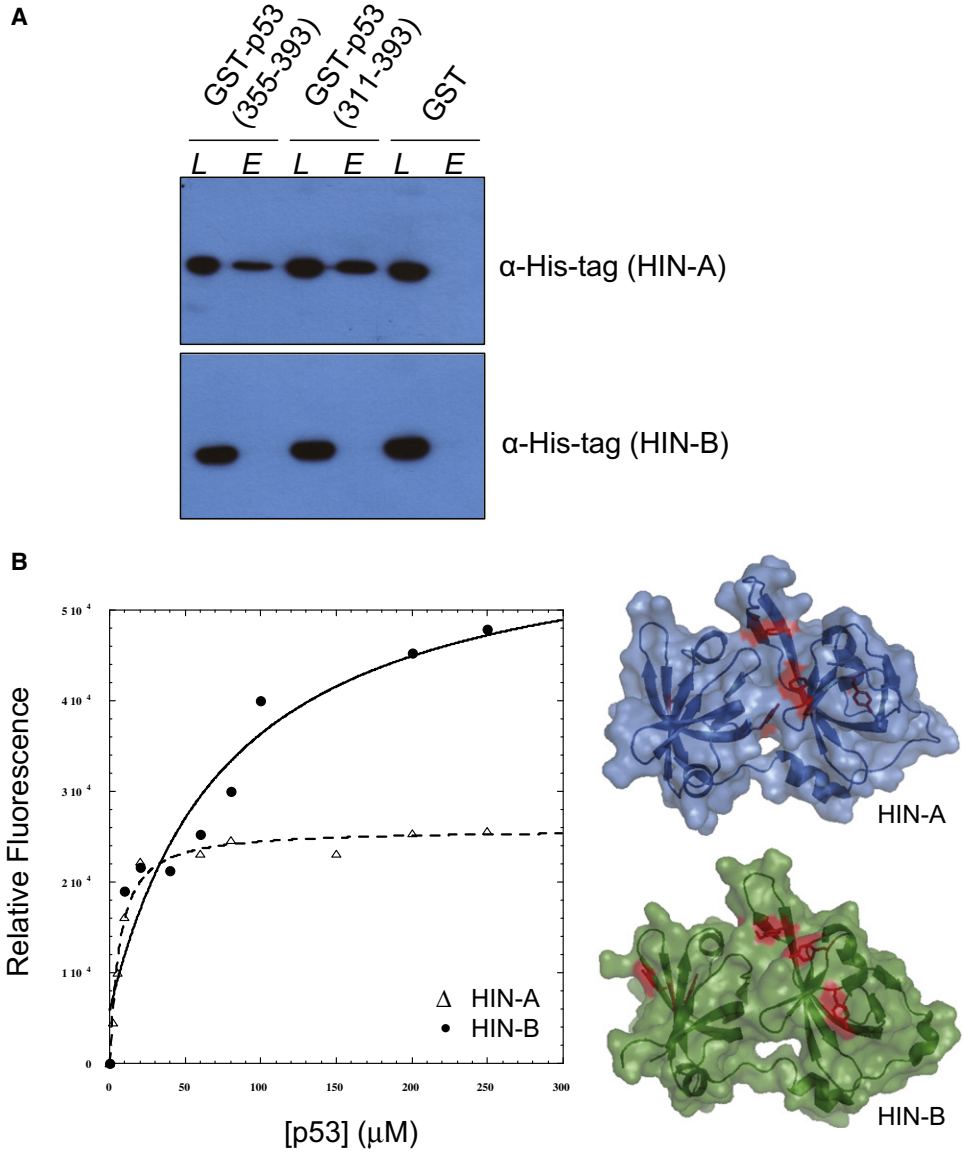

Figure 3. Interaction of p53 C Terminus with IFI16 HIN-A or HIN-B Domain

(A) An equimolar mixture of His-tagged IFI16 HIN-A or HIN-B domain and a GST fusion protein containing the indicated p53 construct was mixed with glutathione-sepharose (*L*). After washing, protein was eluted with glutathione and visualized by western (*E*) using antibody against His-tag.

(B) Increasing amounts of p53 (355–393) were incubated with IFI16 HIN-A (open triangle) or HIN-B (filled circle) domain, and binding was quantified by changes in tyrosine fluorescence. Tyrosine side chains are shown in red on the surface representation of HIN-A and HIN-B crystal structures.

E272A, E222A single mutants (lanes 5–8) were less effective at activation of p53, especially T220A and Y218A. On the other hand, double mutants 222/272 (lane 9) and 218/267 (lane 10), and triple mutant 222/272/381 (lane 11) all abrogated p53 activation completely, suggesting that these residues contribute to the p53 C terminus-binding surface on HIN-A. Circular dichroism measurements indicated that all the mutants were folded and had levels of secondary structure similar to that of wild-type IFI16 (data not shown). Together these six residues form a cluster between strands β2 and β5′ of subdomain 1 and loop L5′5 of subdomain 2, creating the likely p53 C terminus-interacting surface.

IFI16 HIN-B Domain Enhances p53-DNA Binding by Associating with p53 Core Domain

Although HIN-B domain of IFI16 does not interact with p53 C terminus, we turned our effort next to investigate if HIN-B domain could influence instead p53 (residues 82–360) binding to consensus DNA. Employing the polyacrylamide EMSA shown in Figure 6A, increasing amounts (0–80 μM) of HIN-B protein was observed to greatly enhance p53-DNA binding as well as forming higher molecular weight protein-DNA complexes. Next, we asked if HIN-B could associate with p53 (residues 82–360) directly using a protein-protein interaction assay. As shown in Figure 6B, p53 (residues 82–360) can interact weakly

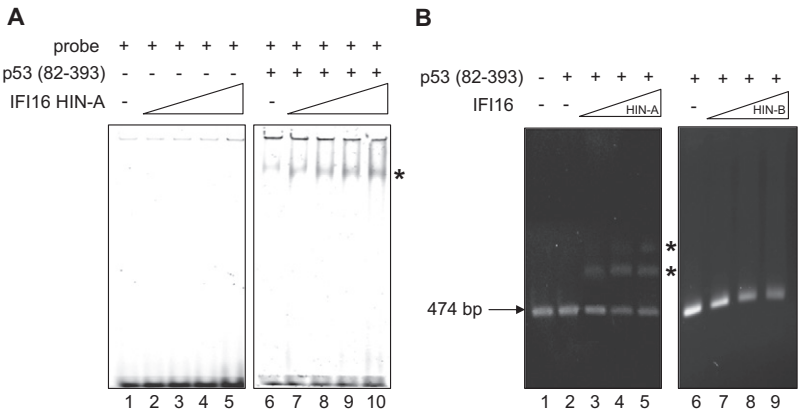

Figure 4. EMSA of p53 (82–393) with p53-Consensus DNA Sequence in the Presence of IFI16 HIN-A Domain

(A) Polyacrylamide EMSA reactions without p53 (lanes 1–5) or with constant amount (20 μM) of p53 (lanes 6–10), in the presence of increasing protein concentration (0, 10, 20, 40, 80 μM) of HIN-A.

(B) Agarose EMSA of constant amount (0.2 μM) of p53 in the presence of increasing protein concentration (0, 0.07, 0.2, 0.33 μM) of HIN-A (lanes 2–5) or HIN-B (lanes 6–9).

with GST-tagged HIN-B, but not GST-tagged HIN-A or GST alone. As presented above, IFI16 HIN-B domain does not interact with the C terminus of p53 (residues 311–393) (Figure 3A), nor does it bind significantly to the 474 bp DNA containing p53-consensus sequence (Figure 4B). Therefore, our data suggest that binding of IFI16 HIN-B domain to p53 may be mediated through the core domain (residues 82–310) of p53, especially when it is already bound to DNA.

Cooperative Effect of Full-Length IFI16 on p53-DNA Binding

Having studied the effects of individual HIN-A and HIN-B domains of IFI16 on p53 sequence-specific DNA binding, it is tempting to ask if the presence of both HIN domains together enhances the activity cooperatively. To address such a question, we purified the 82 kDa full-length IFI16 (residues 1–729) and performed EMSA analysis with p53 (residues 82–393). As seen in Figure 6C, increasing amounts of full-length IFI16 were incubated with a constant amount of p53 and p53-consensus DNA probe. Notably, addition of lowest concentration (0.07 μM) of full-length IFI16 was able to better enhance p53-DNA binding (compare lane 3 of Figure 6C with lane 3 of Figure 4B). This suggests that the presence of both HIN-A and HIN-B domains in the full-length IFI16 protein may cooperatively elevate the effect of individual HIN domains on p53 binding to its consensus sequence. Although full-length IFI16 also possesses a conserved PYRIN domain at its N terminus, this domain was shown by Johnstone et al. (2000) not to interact with p53.

IFI16 Enhances p53-Mediated Transcription Activity

We next examined whether this IFI16-mediated enhancement of p53 sequence-specific DNA binding has an effect on p53 transcriptional activation. We performed luciferase reporter assays to examine the effect of IFI16 on p53-mediated p21 promoter activation. p21 is a functionally important p53-target gene involved in cell cycle control and transcriptional regulation. H1299, a human nonsmall cell lung carcinoma cell line, is used for this study because it is known not to express endogenous p53. In addition we have observed that H1299 cells also did not express endogenous IFI16 (Figure S2). The cells were cotransfected with p53 and/or IFI16 (full-length, HIN-A or HIN-B) together with a luciferase reporter gene containing the 20 bp 5′ response element of p21. As shown in Figure 7, p53 alone, but not IFI16 (full-length, HIN-A or HIN-B), increased the relative luciferase signal by 8-fold. A greater than 15-fold dose-dependent increase in luciferase activity was observed when increasing amounts of full-length IFI16 were cotransfected with p53. Interestingly, when coexpressed with p53, the HIN-A or HIN-B domain of IFI16 was sufficient to promote p53-mediated luciferase activity in a dose-dependent manner, comparable to that of the full-length IFI16. This finding indicates that IFI16, via either HIN-A or HIN-B domain, is capable of upregulating p53-mediated transactivation function.

Full-Length IFI16 Model with p53

IFI16 is involved in transcriptional regulation by modulating protein-DNA and protein-protein interactions with transcription factors such as p53. Using small-angle X-ray scattering (SAXS), an averaged bead model/molecular envelope of the full-length IFI16 protein was derived (Figure 8), with a putative arrangement of its three folded domains, PYRIN (based on homology modeling of PYRIN domain of MNDA, PDB: 2DBG), HIN-A (PDB: 2OQ0), and HIN-B (PDB: 3B6Y) in the bead model. The model adopts a zigzag, elongated overall shape, which is in accordance with the noncompact conformation indicated by the Kratky plot (Figure S3B) and the relatively large Rg value 56 ± 2 Å (Figure S3A). The cross section throughout the bead model is approximately 30–50 Å. In addition the volume of the averaged bead model is 1.95 × 10^5 Å^3, which is in a good agreement with the Porod volume 1.8 ± 0.2 × 10^5 Å^3, estimated directly from the scattering profile. The three domains of IFI16 are oriented relatively independent of each other. In this arrangement, kinks also occur at the junctions between PYRIN/HIN-A and HIN-A/HIN-B at the nonstructured linkers. This model of full-length IFI16 is consistent with its potential function as a scaffold for multiple protein-protein interactions including p53, in which HIN-A and HIN-B domains of IFI16 interact with the C terminus and core domain of p53, respectively. HIN-A may relieve unproductive nonspecific DNA interactions of p53, or otherwise guide the C terminus to facilitate sequence-specific DNA binding by the core domain, whereas HIN-B is likely to interact with DNA-bound core domain stabilizing the p53-DNA complex.

Conclusion

IFI16 plays an important role in regulating cell proliferation and transcription through different protein-protein interactions. We

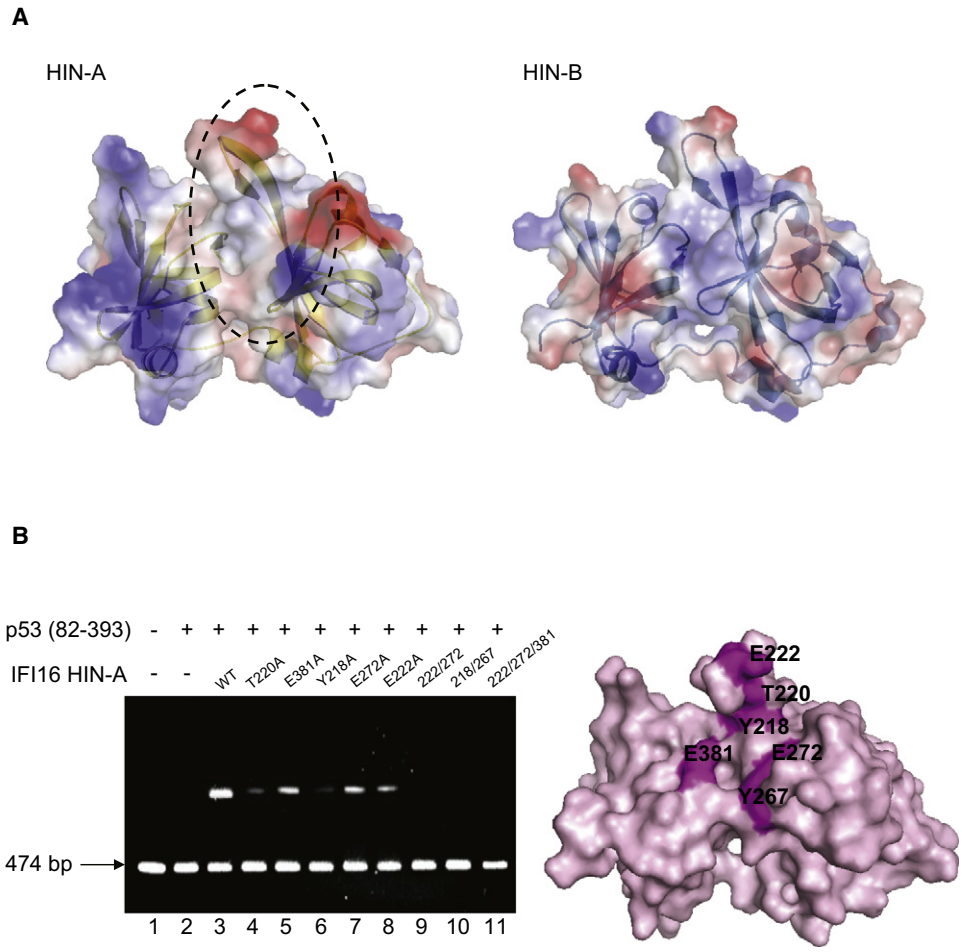

Figure 5. p53 C Terminus-Binding Surface of IFI16 HIN-A Domain

(A) Transparent electrostatic surface representation of IFI16 HIN-A (left) and HIN-B (right) crystal structures. White, red, and blue colors correspond to neutral, negatively, and positively charged surfaces, respectively.

(B) Agarose EMSA of p53 (82–393) with wild-type or mutant IFI16 HIN-A (left). Molar ratio of p53 to HIN-A is 1:3. Surface representation of IFI16 HIN-A (right). Residues involved in binding are colored in magenta.

have solved the crystal structures of the HIN-A and HIN-B domains of IFI16, representing the first experimentally determined HIN domain structures of the HIN-200 family. Despite having low-sequence identity to the OB folds commonly found in various proteins, both HIN domains of IFI16 possess tandem OB folds joined by an α-helical interdomain linker. It is worth noting that the majority of the OB-fold structures reported to date in the Protein Data Bank (PDB) are solitary. Thus, IFI16 joins *mouse* BRCA2 (PDB: 1MIU), *Oxytricha nova* α-TEBP (PDB: 2I0Q), and *human* RPA70 (PDB: 1JMC) to be the only proteins with structural information showing consecutive OB folds. Structural analysis of the human RPA70 has revealed a similar domain organization such that it has four domains (RPA70N, DBD-A, DBD-B, DBD-C) each with an OB fold. The N-terminal OB fold of RPA70 has a role in mediating protein-protein interaction with p53 (Bochkareva et al., 2005), whereas the remaining three OB folds all participate in single-stranded DNA binding (Bochkarev et al., 1997, 2000, 2001). Although the HIN domain of IFI16 constitutes a unique arrangement of tandem OB folds with specific interdomain interactions, it is possible that the four OB folds are also involved in a modular and concerted fashion for multiple substrate recognition analogous to those of RPA70.

The work presented here extends previous studies demonstrating the association of IFI16 HIN-A domain with the C terminus of p53 and further defines the mechanism by which IFI16 stimulates p53-mediated transcriptional activation. Although HIN-A and HIN-B domains of IFI16 are structurally similar and both modulate p53 sequence-specific DNA binding, they interact with p53 differently. IFI16 recognizes the basic p53 C terminus through an acidic/hydrophobic surface present on and unique to HIN-A domain, whereas HIN-B domain associates instead with core domain of p53. It is likely that HIN-A domain prevents p53 from nonspecific DNA interaction via its C terminus, whereas HIN-B domain stabilizes p53-DNA binding. Given its putative role as a scaffold molecule, full-length IFI16 is also likely involved in recruitment of additional factors to the p53-binding sites via its PYRIN domain. Indeed, the BRCA1

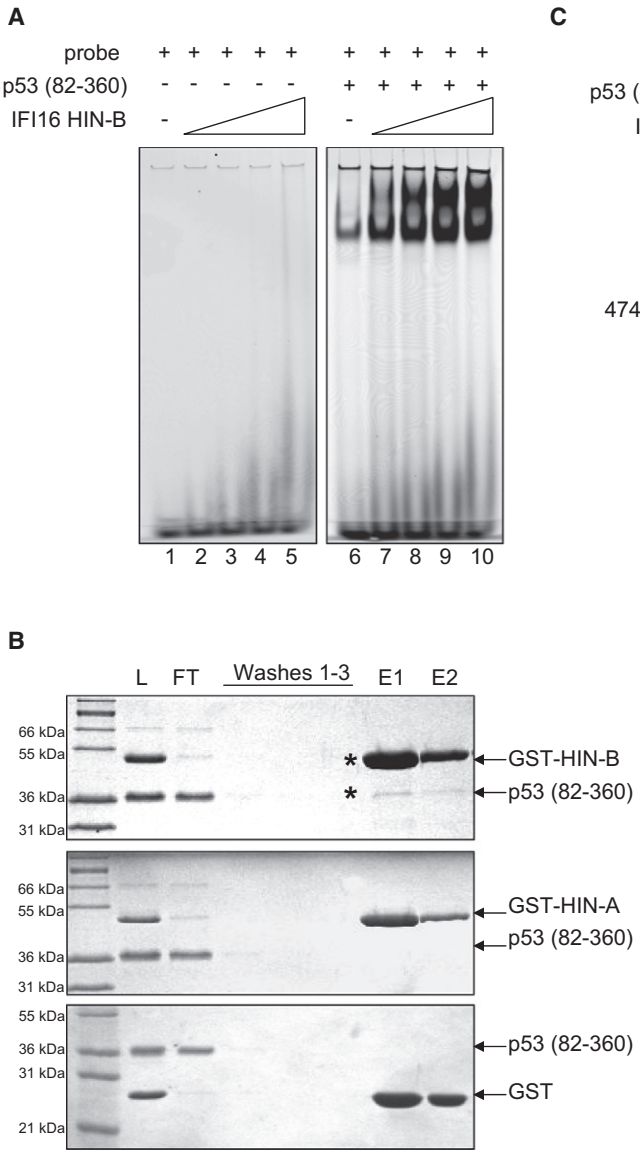

Figure 6. IFI16 HIN-B Domain Enhances p53-DNA Binding by Associating with p53 Core Domain

(A) Polyacrylamide EMSA reactions without p53 (82–360) (lanes 1–5) or with constant amount (2 μM) of p53 (lanes 6–10), in the presence of increasing protein concentration (0, 10, 20, 40, 80 μM) of HIN-B.

(B) GST-pull-down of p53 and HIN-B domain. An equimolar mixture of His-tagged p53 (82–360) and GST-tagged IFI16 HIN-B, GST-tagged IFI16 HIN-A, or GST protein alone was mixed with glutathione-sepharose (L). After washing (Washes 1–3), protein was eluted (E1, E2) with reduced glutathione and visualized by Coomassie blue staining.

(C) Agarose EMSA of constant amount (0.2 μM) of p53 (82–393) in the presence of increasing protein concentration (0, 0.07, 0.2 μM) of full-length IFI16.

tumor suppressor protein is known to interact with IFI16 PYRIN domain and promote p53-dependent apoptosis (Aglipay et al., 2003). Interestingly, we and others have shown that p53 can also associate directly with BRCA1 (Zhang et al., 1998; Mark et al., 2005), thus suggesting intricate crosstalk among the three proteins and also adding another level of complexity in the function and regulation of IFI16.

EXPERIMENTAL PROCEDURES

Expression and Purification

IFI16 gene fragments coding for HIN-A domain (residues 192–393) and HIN-B domain (515–710) were amplified from human IFI16 cDNA (Mammalian Gene Collection, Structural Genomic Consortium) by PCR and subcloned into pET15b (Novagen) expression vector. Both IFI16 domains HIN-A and HIN-B proteins were expressed in *E. coli* BL21-CondonPlus cells (Stratagene) with N-terminal His_6-tag in selenomethionine (Se-Met)-containing media. Protein purification was performed by Co^{2+} affinity column (TALON). Recombinant human p53 protein (residues 355–393, 311–393, 82–360, 82–393) was subcloned into pET15b (Novagen) or pGEX-2TK (Amersham) vector and expressed in and purified from *E. coli* BL21-pLysS cells (Stratagene) as previously described (Ayed et al., 2001; Mark et al., 2005).

Crystallization, Data Collection, and Structure Determination of IFI16 HIN-A

Crystals of the Se-Met-labeled IFI16 HIN-A (35 mg/ml) were obtained by the sitting drop vapor diffusion method by mixing the protein with an equal volume of reservoir solution containing 26% PEG 6000, 0.2 M $MgCl_2$, and 0.1 M Tris-HCl at pH 8.5. The crystals belong to the monoclinic space group $P2_1$ with unit cell parameters of a = 43.3 Å, b = 88.9 Å, c = 112.8 Å, and β = 99.4°. For data collection and structure determination details, please refer to Supplemental Experimental Procedures.

Crystallization, Data Collection, and Structure Determination of IFI16 HIN-B

Native crystals of IFI16 HIN-B (23 mg/ml) were obtained by the sitting drop vapor diffusion method by mixing the protein with an equal volume of reservoir solution containing 26% PEG 4000, 0.2 M NH_4SO_4, and 0.1 M sodium cacodylate buffer at pH 6.5. The crystals belong to the orthorhombic space group $P2_12_12_1$ with unit cell parameters of a = 43.0 Å, b = 92.9 Å, and c = 100.3 Å with two molecules in the asymmetric unit. For data collection and structure determination details, please refer to Supplemental Experimental Procedures.

Protein-Protein Interaction Assays

Purified GST, GST-p53 (residues 355–393), or GST-p53 (residues 311–393) fusion protein was first mixed with His-tagged IFI16 (residues 192–393) or His-tagged IFI16 (residues 515–710) protein in assay buffer (20 mM HEPES [pH 7.6], 150 mM NaCl) and then incubated with the glutathione beads (Amersham) at 4°C for 2 hr. After extensive washing with assay buffer, bound proteins were eluted with 30 mM reduced glutathione and detected after SDS-PAGE by western analysis using antibody against His-tag (QIAGEN). Similar assays were conducted for purified GST, GST-HIN-A (residues 192–393), or GST-HIN-B (residues 515–710) fusion protein with His-tagged p53 protein (residues 82–360).

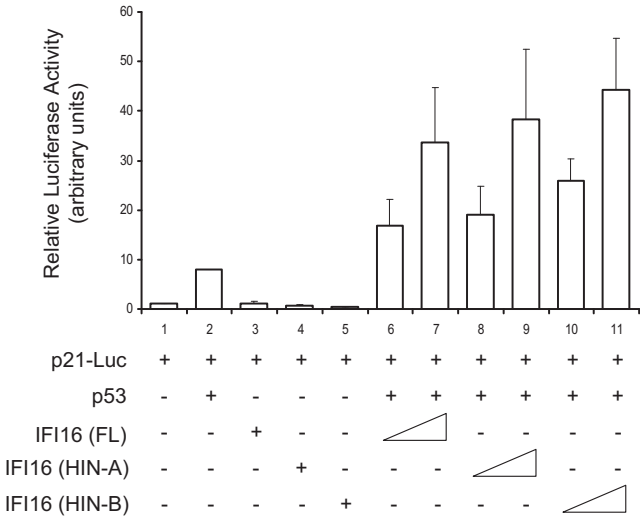

Figure 7. IFI16 Enhances p53-Mediated Transcription of the p21 Promoter

H1299 cells were cotransfected with the luciferase reporter vector containing the full-length p21 promoter, p21-Luc, and with pcDNA3-p53 and/or pcDNA3-IFI116 (full-length [FL], HIN-A or HIN-B). The cells were lysed and assayed for luciferase activity 48 hr after transfection. Standard error was calculated using three independent experimental readings. See also Figure S2.

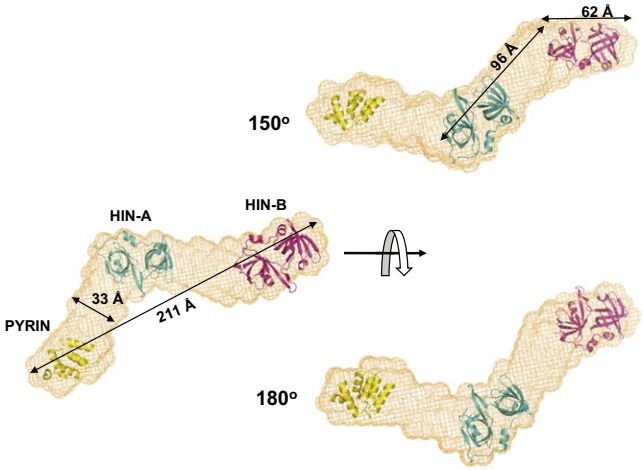

Figure 8. Model of Full-Length IFI16

An averaged bead model/molecular envelope of the full-length IFI16 calculated from SAXS data, with dimension measurements and putative arrangement of its three folded domains: PYRIN, HIN-A, and HIN-B. See also Figure S3.

Intrinsic Tyrosine Fluorescence Assay

Direct binding of p53 C terminus (residues 355–393) to IFI16 HIN-A domain (residues 192–393) or HIN-B domain (residues 515–710) was characterized based on principles employed by Leclerc et al. (1993), Gupta et al. (2008), and Yan et al. (2008). A p53-binding titration was performed manually by mixing a solution of IFI16 protein (final concentration 1 μM) with increasing amounts of p53 (0–250 μM) in a buffer containing 25 mM Tris-HCl (pH 8.0) and 75 mM NaCl in a total volume of 150 μl at room temperature. After mixing, the intrinsic tyrosine fluorescence intensity (I) was measured using a time-based experimental module, which averages the fluorescence intensity from 30 readings with each reading for 1 s and sets the excitation wavelength at 278 nm, emission wavelength at 307 nm, and bandwidth at 4 nm. The quenched fluorescence (I_q) was calculated by $I_o - I$, in which I_o was the fluorescence intensity of IFI16 protein without p53. Background signals from the buffer and ligand were corrected. The dissociation constants (K_d) were determined by fitting the binding curve to Equation 1, using KaleidaGraph Version 3.0 (Abelbeck Software):

$$I_q = I_o + \frac{(I_\infty - I_o)(1 + [P]/k_d + [L]/k_d) - \sqrt{(1 + [P]/k_d + [L]/k_d)^2 - 4[P][L](1/k_d)^2}}{[L](2/k_d)}, \quad (1)$$

where I_q is the fluorescence intensity at a given total concentration of target ligand, [L]. [P] is the total concentration of protein in the reaction. K_d is the dissociation constant. I_∞ is the fluorescence intensity at saturation, and I_o is the fluorescence intensity in the absence of ligand.

Polyacrylamide EMSA

p53 (2–20 μM) was incubated with double-stranded DNA probe 5′-GGACATGCCCGGGCATGTCC-3′ (1–2 pmol) tagged with the monoreactive fluorescent reagent Cy5-Dye (Sigma) in EMSA buffer (20 mM Tris-HCl [pH 6.8], 150 mM NaCl, 10% [v/v] glycerol, 5 mM DTT) in the presence of IFI16 HIN-A or HIN-B protein (at final concentrations of 0–80 μM). Complexes were resolved by electrophoresis at 100 V at room temperature using a pre-run 5% polyacrylamide gel containing Tris-Borate-EDTA buffer and then visualized by red fluorescence emission measured at 635 nm using a STORM860 scanner (Molecular Dynamics).

Agarose EMSA

DNA (600 ng of PvuII fragments of pPGM2) (Brazda et al., 2006; Jagelska et al., 2008), p53, and IFI16 were mixed at various ratios in 20 μl of the DNA-binding buffer (5 mM Tris-HCl [pH 7.0], 1 mM EDTA, 50 mM KCl, and 0.01% Triton X-100). The samples were incubated for 10 min at 4°C and loaded onto a 1% agarose gel (SERVA) containing 0.33× Tris-Borate-EDTA buffer. Agarose electrophoresis was performed for 4 hr at 100 V at 4°C. The gels were stained with ethidium bromide and photographed.

Cell Culture and Luciferase Assay

Human nonsmall cell lung carcinoma cell line H1299 was obtained from the Benchimol laboratory and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). pcDNA3-p53 and the luciferase reporter construct carrying the p21 promoter (p21-Luc) were also obtained from the Benchimol laboratory. An amplified fragment of full-length IFI16, HIN-A, or HIN-B from human IFI16 cDNA (Mammalian Gene Collection, Structural Genomic Consortium) was subcloned into pcDNA3 (Invitrogen). H1299 cells at ~80% confluence in a 6-well plate were transfected using Lipofectamine 2000 (Invitrogen). At 48 hr after transfection, luciferase assay was performed using the Luciferase Assay Kit (Promega) and measured using the SpectraMax M5 luminometer (Molecular Devices). Standard error was calculated using three independent experimental readings.

SAXS Experiments, Data Analysis, and Bead Model Reconstruction

Both SAX and wide-angle X-ray scattering (WAXS) were performed at beamline 12-ID of the Advanced Photon Sources (APS) at the Argonne National Laboratory. A detailed description about experimental procedure, processing and analysis of scattering data, and bead model reconstruction can be found in Supplemental Experimental Procedures.

ACCESSION NUMBERS

Atomic coordinates and structure factors for the reported crystal structures have been deposited in the PDB under the accession codes 2OQ0 and 3B6Y.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at doi:10.1016/j.str.2010.12.015.

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