# Peptide-*N*-glycanases and DNA repair proteins, Xp-C/Rad4, are, respectively, active and inactivated enzymes sharing a common transglutaminase fold

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Yeast RAD4, its human ortholog Xp-C and their orthologs in other eukaryotes are DNA repair proteins which participate in nucleotide excision repair through a ubiquitin-dependent process. However, no conserved globular domains that might have shed light on their origin or functions have been reported for these proteins. By using sequence profile analysis, we show that RAD4/Xp-C proteins contain the ancient transglutaminase fold and are specifically related to the recently characterized peptide-N-glycanases (PNGases) which remove glycans from glycoproteins during their degradation. The PNGases retain the catalytic triad that is typical of this fold and are predicted to have a reaction mechanism similar to that involved in transglutamination. In contrast, the RAD4/Xp-C proteins are predicted to be inactive and are likely to only possess the protein interaction function in DNA repair. These proteins also contain a long, low-complexity insert in the globular transglutaminase domain. The RAD4/Xp-C proteins, along with other inactive transglutaminase-fold proteins, represent a case of functional re-assignment of an ancient domain following the loss of the ancestral enzymatic activity.

# INTRODUCTION

Eukaryotes have a complex system of nucleotide excision repair (NER) (1,2) that has been the subject of extensive studies, particularly in connection with the inactivation of various components of this system in human diseases such as Xeroderma pigmentosum [XP (3)]. Studies with the yeast model system and with XP complementation groups have led to the identification of a variety of repair enzymes, including DNA helicases and ATPases, such as the ERCC2/3, and nucleases, such as ERCC1/XP-F and XP-G. Additional components of the excision repair system include Xp-C/Rad4, RAD23 and RAD7 that mediate DNA–protein and protein–protein interactions. Most of these proteins contain recognizable, conserved globular domains which are consistent with the corresponding biochemical activities (4,5). Yeast Rad4 and its human ortholog XP-C play an important role in the recognition of DNA damage and recruitment of the TFIIH complex for excision repair (6–9), but contain no previously identified domains. Here we show that Rad4/XP-C is an inactive homolog of the recently identified peptide-*N*-glycanases (PNGases) that are involved in glycoprotein degradation (10) and that these proteins share a core transglutaminase fold.

# **RESULTS AND DISCUSSION**

Iterative searches of the non-redundant protein sequence database (National Center for Biotechnology Information, NIH, Bethesda) using the PSI-BLAST program (11) revealed statistically significant sequence similarity between the Xp-C/Rad4 proteins and the PNGases. These searches also showed a statistically significant similarity between the Xp-C/Rad4, PNGases and the proteins of the transglutaminase superfamily (12) (Fig. 1). PFAM search tools that utilize Hidden Markov Models based on alignments from the PFAM database (13) also identified the transglutaminase domain in the yeast Rad4 and PNGases (E-values: 10-4-10-3), but not in XP-C or other RAD4 orthologs. The presence of the transglutaminase fold in these proteins was further confirmed by carrying out sequence-structure threading using the hybrid fold recognition method (14), with the yeast Rad4 as a query. This resulted in the detection of PDB:1FIE as the best hit. The transglutaminase superfamily includes, in addition to the well-characterized transglutaminases such as the vertebrate clotting-factor XIIIA', several proteases and many uncharacterized proteins that are found in a broad range of prokaryotes and eukaryotes (12). The majority of the proteins of this superfamily are known or predicted to be active enzymes that utilize a catalytic triad comprised of a histidine, a cysteine and an aspartate (Fig. 1) and resembling the active site of papain-like proteases (12,15). The reactions catalyzed by these enzymes involve either the formation of amides by linking alkylamines to the glutamate side chains of proteins or hydrolysis of peptide bonds in the case of proteases (12,15). Thus, the finding of the transglutaminase fold in the PNGases is consistent with the reaction catalyzed by these enzymes that involves breakage of the amide bond between N-acetylglucosamine and an asparagine side chain (10). This is confirmed by the inactivation of the

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Secondary structure		
Ky Mm 10697088	220	1
CG13435 Dm 7302360	262	(
CG13435_Dm_7302360 K02C4.4_Ce_7505121 YEB8 Sp_2370554	385	¢
YEB8_Sp_2370554	531	ł
	519	Ē
sll1681 Ssp 1651866	519 193 208 193	ł
peiP psiM2 3249613	208	1
peiW Mw 11863120	193	1
peiW_Mw_11863120 DR0843_Dr_7471673 sll1233_Ssp_1653166 AAC08420_Scsp_2996036	182	ŝ
s111233 Ssp 1653166	177	1
AAC08420 Scsp 2996036	175	\$
RV2569C_MC_1478243	191	1
MLCB1259.25_M1_3136019	430	ł
Rv2409c_Mt_1655650	170	4
VD60 M1 2406516	170	ŝ
4.2 Hs 112798	263	٦
Xiii_Hs_1942386	309	١
TGLC_Hs_135693	272	1
4.2 Hs 112798 Xiii Hs 1942365 TGLC_Hs_135693 TGLK_Hs_2895530 TGLE_HS_7433818 TGLK_Hs_401177 PNGase_Ms_8347619 PNGase_Hs_8347614 PNGase_Cc_8347617 PNL096% 52 2132186	273	١
TGLE Hs 7433818	268	١
TGLK_Hs_401177	372	٦
PNGase_Dm_8347619	291	1
PNGase_Hs_8347614	223	1
PNGase Ce 8347617	182	1
SPBC1709.14_Sp_7490317	158	ŝ
K6M13.12 At 10177623	246	1
Rad4 Sc 131813	255	٦
YDR314C Sc 6320520	255 231	ł
SPCC4G3.10C Sp 7491911	259	- 1
SPAC12B10.12C Sp 7492535	189	1
Y76B12C.2 Ce 7105677	513	1
SPAC12B10.12C Sp 7492535 Y76B12C.2 Ce 7105677 XPC Dm 3915300 XPCC He 1722884	472	Į
XPCC_Hs_1722884	288	ł
consensus/90%		

condary structure		eeehhhHHH.HHHHHHHheEEEEEEeeeeeEEEEEEEeeEEEEE	
Mm 10697088	220	TQKT-NCDGYAG-LFERMCRVAGYQCVTYPGYSKGFGYQTGQSFSG-EFDHANNAYYLEGRLDLLYNE 284\	
13435 Dm 7302360		GIKY-GTESYHV-LFKRLCSYAGLHCVVIKGYSKSAGYQPGVKFQDS-RFRNSWNAVYVAGAMREVQCN 327	
2C4.4 Ce 7505121		GIKY-GTESYHV-LFKRLCSYAGLHCVVIKGFSKSAGYOPGYSF	
B8_Sp_2370554		EGQG-TPFEVAL-LVKEMLQALDLWCEVIEGYLKSPDDIYYT	/
k3_Sc_1431172		RKHC-TPYELTW-LFKKLANSLGITCEIVIGFLKTPSAIN	
11681 Ssp 1651866		RGET-ISSYNN-LYOALAKELGLUVVIIEGFAKGGDVIVG	
iP psiM2 3249613		TDGI-N <mark>CTDACQ-L</mark> FKP <mark>VIEGLGY</mark> SVR <mark>I</mark> EHVKVRCN	
iW_Mw_11863120		TSGI-NOTDACQ-LFSKVLEEMGYEVKIEHVRVKCN	
0843_Dr_7471673	182	SGRA-V <mark>ERDEAH-M</mark> GVA <b>FCRALNIFARYV</b> CGYMPDIDYPD	
11233 Ssp 1653166		SREG-SERDLTV-LFMEVCRAMGLARFVSGYEVGDPE	
C08420 Scsp 2996036	175	ORTG-TORDEAL-LAWEACRVAGLAAREVSGYORGDET	
2569c Mt 1478243	191	AREG-VOODEAR-LAIACLRANGLAACYVSGYLATDPPPGKDRM	
CB1259.25_M1_3136019		AREG-V <mark>QQD<b>F</b>AR-<mark>L</mark>AIA<b>CL</b>RANG<b>L</b>AAS<mark>YV</mark>SGYLATDPPPGKDRM</mark>	
2409c Mt 1655650	170	QGKG-V <mark>QQD<b>F</b>VH-LSLMVLRSMGTPCRYVSGYLHPKRDAVVGK</mark>	
69_M1_2496516	170	QCRG-V <mark>QQDEAH-L</mark> TLIVLRSMGIPGRYYSGYLHPKRDAVVGK	
2 Hs 112798	263	VYDG-QAWVLAA-VACTVLRCLGIPARVVTTFASAQGTGGRLLIDEYYNEEGLQNGEGQRGRIWIFQTSTECWMTRPALPQG-YDGMQILDPS 352\	
ii_Hs_1942386	309	VRYG-OGWVEAG-VFNTELRCLGIPARIVINYFSAHDNDANLOMDIFLEEDGNVNSKLTKDSVWNYECWNEAMMTRPDLPVG-FGGMOAVEST 398  Classic	
LC Hs 135693	272	VKYG-QOWVEAA-VACTVLRCLGIPTRVVINYSAHDQNSNLLIEYFRNEFGEIQGDKSEMIWNFICHVESWMTRPDLQPG-YEGWQALEPT 360  Trans-	
LX_Hs_2895530	273	VRYG-QOWVEAA-WMCTVMRCLGIPTRVITNFDSGHDTDGNLIIDEYYDNTGRILGNKKKDTIWNFEVWNECKMARKDLPPA-YGGMQVLEAT 362  glutaminases	
LE_Hs_7433818	268	VRYG-Q <mark>enve</mark> ag- <mark>z</mark> lnt <b>alrslgzpsrvz</b> tnensahdtdrnlsvdvyydpmgnpldkgsdsvwne <mark>nvn</mark> neg <mark>w</mark> evrsdlgpp-ygg <mark>w</mark> gv <mark>ls</mark> at 356	
LK Hs 401177		VPYG-QCWVFAG-VITTGLRCLGLATRIVINFNSAHDIDTSLIMDIYFDENMKPLEHLNHDSVWNFEVNNDCWMKRPDLPSG-FDGMQVVEAT 461/	
Gase_Dm_8347619		SRKG-R <mark>e</mark> ge <b>y</b> an-cftf <b>lcraldydariv</b>	
Gase_Hs_8347614		TRCG-REGEMAN-CFTLCCRAVGFEARYVQQRMLHCEAC 274	
Gase_Ce_8347617	182	TRTG-R <mark>SGEWAN-C</mark> FGL <b>LLAAINLESRFI</b>	
L096w_Sc_2132186	186	TRKG-REGEWCN-LFTLILKSFCLOVRYVNNREDEVWCEYFSNFLNRWVHVESC 237	
BC1709.14_Sp_7490317	158	SRKG-R <mark>SCEWAN-C</mark> FTF <b>LCRALG</b> SRARWIOORMVHVDSG 209	
M13.12_At_10177623		TKKG-REGENAN-CFTLYCRTFGYDSRLI	
d4_Sc_131813	255	VSKGHGDPDISVCGFVAMLRACNYUARLIMSCQP-PDFTNMKIDTSLNGN NAYKDNVKYPIFMCEVNDKFSKKWITVDPV 333\	
R314C_Sc_6320520	231	KRK-MANRD <mark>i</mark> lti <mark>r</mark> ffi <mark>ilenv</mark> leg <b>pkku</b> ylcfalplhdydircnkvkmqiehgigkvp- nrfdsdliqpy <b>fwiel</b> evptlsdge <mark>l</mark> yi <b>i</b> dppi 320	
CC4G3.10c_Sp_7491911	259	LLSLKGSRDLAAQGFTALCRSLNLKARLIFSLQPLTFSTASYDDWSPHILPEETSTSIDDDLRYPIFWTEIYDQSEKKWIAVDAV 343	
AC12B10.12C_Sp_7492535	189	SKLLSG <b>SRDY</b> GTQ <mark>L</mark> FAS <mark>ILRNLNY</mark> PTRLYFSLQVLSFRFKGAINEASSHEIVPAWSQQME 44 KLKVIDSPKPV <b>FW</b> VE <b>AF</b> NKAMQKWVC <mark>V</mark> DPF 322  RAD4/XPC	
6B12C.2_Ce_7105677		NRKIREMNENTHK <mark>l</mark> ifC <b>lirgLeittrivvnvraiprrwdktookelonelskfrelsrs 23 aakkvvveern<mark>iw</mark>ve<mark>iw</mark>oprekr<mark>w</mark>ic<mark>v</mark>opl 635  </b>	
C_Dm_3915300		KRKEARCKODMIF <mark>I</mark> FIA <b>LARGMGMHCRLI</b> VNLOPMPLRPAASDLIF <u>I</u> KLRPDDKNKSOTV 417 SRLNRKTDASD <mark>MMVEVM</mark> SDVECOMICIDLF 978	
CC_Hs_1722884	288	AIYSARDDEELVH <mark>I</mark> FLL <mark>ILRALOL</mark> LTR <mark>LV</mark> LSLQPIPLKSATAKGKK <mark>S</mark> SKERLTADPGGSS 171 KAEKRSIAGIDQNLE <mark>VF</mark> CEQEEK <mark>N</mark> VC <mark>V</mark> DCV 548/	
nsensus/90%		shhwshsh.sphlWhDs.	

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Seconda PNGase\_I PNGase PNGase YPL096 SPBC170 K6M13.1 Rad4\_Sc YDR3140 SPCC4G3 SPAC12E Y76B12C XPC\_Dm XPCC H нÌ

ary structure			e	eEe		EEEEE	E	HhH	HHHHh		hhhHH			HHHH	HHHH.	HHE.		HHHH	ЕННИНИ	h			
Dm_8347619	343	ENV	IDSPL:	<b>IYQHGWK</b>	RH	IDYIL	YSRD	DIQDY	TWRYT		NDHQK	ILHLR	KL/CG=-	EKEN	TOVN	DAI	-RAKRRQ	NCTADR	KLFLSQR	IVBYNN	429\		
Hs 8347614	275	EDV	CDKPLI	YEIGWG	KK	LSYVI	FSKD	EVVD	VTWRY S	5	CKHEE	VIARR	TKVK	EAL	LRDTI	NGL	-NKQRQI	FLSENR	RKELLQR:	IIVELV	361	1	
Ce 8347617	234	ENT	MDRPLI	YTRGWG	KT	LGYCI	YGSD	HVVD	VTWR <mark>Y</mark> I		WDSKK	LVTQR	NEVR	QPV	PENFL	SKL	-NSRQAE	GQTEPR	KRELAVR	RVCELM	320	PNGase	
w Sc 2132186	238	EQS	FDQPY:	YSINWN	KK	MSYCI	FGKD	G <mark>V</mark> VD <mark>V</mark>	VSKRY I		LQ	NELPR	DQIK	EEDI	LKFLC	QFI	-TKRLRY	SLNDDE	IYQLACRI	DEQEQI	321	1	
09.14 Sp 7490317	210	EES	FDEPLI	YEQGWG	KK	MSYCL	FGID	SVRDV	VSHRY I		RHPEN	GL-PR	DRCP	ESVI	LOOAL	HEI	-NIEFRS	RLTDSE	RKALEEEI	DKREKD	295	1	
12 At 10177623	298	EGV	ZDKPMI	YEKGWN	KK	LNYVI	ISKD	GVCDV	<b>VT</b> KR <b>Y</b> T		KKWHE	VLSRR	TLTT	ESS <mark>I</mark>	LODGL	RTL	TRERRRSI	MFESLS	KLELRDR	NEQEEL	386/		
c_131813	334	-NLKT	<b>IBQV</b> RI	HSKLAP	KG 8	LRYVI	YDRKY-	GCRD	VTRRYA	1	WMNSK	VRKRR	ITKDDI	F-GEKW	FRKVI	TAL	-HHRKRT	KIDDYE	DQYFFQRI	DESEGI	434\		
C Sc 6320520	321	AHLGE	REMVLE	TREDQF	VP 15	FHYVV	INHAEK	VLQDV	VSPRYV	10	SESSP	ILKSK	HYTS	YQY	LSKWL	KVT.	-NKKKA	SVHHYA	IMKKIAL	INFTLP	435	1	
3.10c Sp 7491911	344	VLNGV	TNDM	WFEPKG	AY 7	MGIVA	YDNDL-	YAKD	VTLRY I	1	YQSSR	LKKIR	HVSFAL	DKYFDF <mark>y</mark>	Y KAI F	GQL	-AKRNK	DAE	DIYEEKEI	LESKVP	441	1	
B10.12C Sp 7492535	323	GD	ASVIGE	YRRFEP	AS 6	MTYVE	IEANG-	Y <mark>V</mark> KD <mark>y</mark>	VTRKY C	n.	LHYYK	ILKNR	VEIFP-	FGKAW	MNRIF	SKI	-GKPRDFYN	DMDAIE	DAELLRL	EQSEGI	420	RAD4/XPC	
C.2 Ce 7105677	636	HKS	VDEPLS	SIHEHSA	SP	ISYVE	IDNKQ-	GICEN	VSQR <mark>Y</mark> A	1	DCVKQ	DFRRR	RTNP	-KWVAWI	<b>TLFLP</b>	PFA	ANSERK	KWE	MMQMREDI	LVKRPL	723		
3915300	979	KGK	LHCVD1	TIRKNAT	PG	LAYVE	FODDQ-	SLKD	TARYC		-ASWS	TTVRK	ARVE	KAWI	DET	APY	LGRRT	KRDITE	DDQLRRI	HSDKPL	1064	1	
s 1722884	549	HGV	VGQPL1	CYKYAT	KP	MTYVV	IDSDG-	WVRDV	TORYD		-PVWM	TVTRK	CRVD	AEWN	AETL	RPY	QSPFM	DREKKE	DLEFQAK	HMDQPL	634/		
sus/90%			hp.s.			h.Yhhu	hp	.h.DV	/o.RY.			+		h	1h	h.		p.		p			

Figure 1. Multiple alignment of the Rad4/XP-C and PNGase sequences with previously identified members of the transglutaminase superfamily. (A) Alignment of the transglutaminase core domain. (B) Alignment of the C- terminal extension specific to the XP-C/Rad4 and PNGase-like proteins. The different families are denoted on the right. A PSI-BLAST search started with the yeast PNGase (PNG1p) sequence, with a profile inclusion threshold of E = 0.01, revealed a shared conserved region with the Rad4/XP-C proteins (for example, RAD4p was detected in this search with an E-value of 10-6 in the second iteration). Further search iterations with this region allowed the detection of several transglutaminase family members including factor XIIIA' for which a crystal structure is available (E-value 10<sup>-4</sup> in the seventh iteration). The multiple alignment was constructed using ClustalW, followed by manual adjustment on the basis of PSI-BLAST search results and secondary structure predictions. The secondary structure shown above the alignment is derived from the crystal structure of factor XIIIA' (PDB: 1FIE) for the transglutaminase core domain and predicted using the PHD program (26) for the C-terminal extension specific to the XP-C/Rad4 and PNGase-like proteins. The 90% consensus shown below the alignment was derived using the following amino acid classes: hydrophobic (h: ALICVMYFW, yellow highlight); the aliphatic subset of these (I: ALIVMC, yellow highlight); alcohol (o: ST, blue), small (s: ACDGNPSTV, green), the 'tiny' subset of these (u: GAS, green highlight), polar (p: CDEHKNQRST, violet), positively charged (+: HKR, pink); charged (c: DEHKR, pink). Black shading indicates the position of mutation in Xeroderma pigme tosum (28,29). The residues of the catalytic triad are shown in reverse shading (shaded in red with yellow letters) in those proteins that retain all three of them. The numbers on each side indicate the limits of the conserved domain in the corresponding protein sequences. The numbers within the alignment are inserts that are not shown. The sequences are denoted by their gene name followed by the species abbreviation and GenBank identifier. The species abbreviations are: Dr, Deinococcus radiodurans; Ml, Mycobacterium leprae; Mt, Mycobacterium tuberculosis; Scsp, Synechococcus PCC7002; Ssp, Synechocystis PCC6803; Mw, Methanothermobacter wolfeii prophage psiM100; PsiM2, Methanobacterium phage psiM2; At, Arabidopsis thaliana; Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster; Hs, Homo sapiens; Mm, Mus musculus; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe.

yeast PNGase by a mutation that eliminates the cysteine of the transglutaminase fold catalytic triad (Fig. 1) (10).

The RAD4/XP-C proteins are the closest homologs of the PNGases within the transglutaminase superfamily; these two groups of proteins share a unique feature, a conserved C-terminal extension, to the exclusion of all other members of this superfamily (Fig. 1). All animal Rad4/XP-C proteins and one of the paralogs from Schizosaccharomyces pombe contain a large, compositionally biased insert between strands 2 and 3 of the transglutaminase fold (Fig. 1). Examination of the multiple alignment shows that RAD4/XP-C proteins lack the (predicted) catalytic residues, suggesting that these proteins emerged early in the evolution of eukaryotes through a duplication of the PNGase, followed by elimination of the enzymatic activity due to the disruption of the active site triad (Fig. 1). At least two other cases of similar, independent, secondary inactivation of the transglutaminase superfamily proteins following their divergence from active ancestors were noticed, namely the erythrocyte protein-band 4.2 and a highly conserved family of potential cytoskeletal proteins, typified by mouse Ky protein (16) and yeast Cyk3 protein (17) and represented in eukaryotes and cyanobacteria (Fig. 1). Due to the general ability of the ancestral forms of these enzymes to interact with other proteins, some of their inactive descendants probably have been recruited for a non-catalytic interaction function. Notably, the PNGases are required for the early stage of proteasomal degradation of glycoproteins (10), whereas Rad4 has also been shown to interact with the proteasome via the atypical ubiquitin homolog, Rad23 (18,19). Thus, it appears that Rad4/XP-C has evolved from an ancestral N-deglycosylase or protease that could have been involved in proteasome-dependent degradation of chromosomal proteins. With the recruitment of the ubiquitin-dependent machinery for a non-proteolytic function in NER, Rad4 could have been recycled to function as an adaptor in protein-protein interactions that are required for this form of repair.

The inactive transglutaminase is the only globular domain (20) in the RAD4/Xp-C proteins, which makes this domain a candidate for the role of the determinant of the specific protein–protein interaction of these proteins. Consistent with this, the portion of RAD4 that encompasses the tranglutaminase domain is required for the interaction with the leucine-rich-repeat containing protein RAD7 (21).

Recruitment of inactivated proteases has been described as one of the evolutionary sources of eukaryotic transcription factors (22). The present observations indicate that evolutionary exaptation (recycling of proteins that have lost their original activity) of the transglutaminase fold for protein–protein interaction has occurred on several independent occasions in contexts as different as NER complex and cytoskeletal organization.

## MATERIALS AND METHODS

The non-redundant database of protein sequences (National Center for Biotechnology Information, NIH, Bethesda, MD) was searched using the BLASTP program (11). Profile searches were conducted using the PSI-BLAST program with either a single sequence or an alignment used as the query, with a profile inclusion expectation (E) value threshold of 0.01, and were iterated until convergence (11,23). Previously known, conserved protein domains were detected using the corresponding position-specific scoring matrices, which were constructed using PSI-BLAST (24). Multiple alignments of protein sequences were constructed using the ClustalW program (25) and protein secondary structure was predicted using a multiple alignment as the input for the PHD program (26). Structure visualization was done with Swiss PDB Viewer (27). Sequence-structure threading was performed using the hybrid fold prediction method that combines multiple alignment information with secondary structure prediction (14).

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