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Molekulární a funkční charakterizace receptoru DR6
Molecular and functional characterization of the death receptor 6

Disertační práce

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Prohlášení:

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V Praze, 26. dubna 2011

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Martin Klíma

Děkuji všem, kteří mi umožnili vypracování této práce, za jejich podporu, pomoc a trpělivost.

Obsah

Seznam použitých zkratek

ADAM	protein obsahující disintegrinovou a metalloproteázovou doménu *a disintegrin and metalloproteinase domain protein*
AICD	buněčná smrt indukovaná buněčnou aktivací *activation induced cell death*
ALPS	autoimunitní lymfoproliferativní syndrom *autoimmune lymphoproliferative syndrome*
Apaf-1	faktor aktivující apoptotické proteázy 1 *apoptotic protease activating factor 1*
APLP	protein podobný prekurzoru proteinu amyloid-β *amyloid-β precursor-like protein*
APP	prekurzor proteinu amyloid-β *amyloid-β precursor protein*
ARAP	protein obsahující domény Arf-GAP, Rho-GAP, ankyrinové domény a PH *Arf-GAP, Rho-GAP, ankyrin repeat and PH domain-containing protein*
BAFF	faktor aktivující B-lymfocyty *B cell-activating factor*
Bcl-2	protein B-buněčných lymfomů 2 *B-cell lymphoma 2*
BCMA	faktor pro zrání B-lymfocytů *B-cell maturation factor*
BH	Bcl-2 homologní doména *Bcl-2 homology domain*
CAD	kaspázami aktivovaná deoxyribonukleáza *caspase-activated deoxyribonuclease*
CARD	doména rekrutující kaspázy *caspase recruitment domain*
CARP	protein příbuzný proteinu karbonická andhydráza *carbonic anhydrase-related protein*
CD	názvoslovná jednotka leukocytárních antigenů *cluster of differentiation*
cIAP	buněčný inhibitor apoptózy *cellular inhibitor of apoptosis*
CRD	doména bohatá na cystein *cystein rich domain*
CTLA-4	cytotoxický antigen T-lymfocytů 4 *cytotoxic T-lymphocyte antigen 4*
CYLD	gen pro cylindromatózu *cylindromatosis gene*

DcR	tlumivý receptor *decoy receptor*
DD	doména smrti *death domain*
DED	efektorová doména smrti *death effector domain*
DFF45	faktor fragmentace DNA, 45kDa *DNA fragmentation factor, 45kDa*
DIABLO	protein s nízkým pI, který přímo váže inhibitory apoptózy *direct IAP-binding protein with low pI*
DISC	signalizační komplex indukující buněčnou smrt *death inducing signaling complex*
DNA	deoxyribonukleová kyselina *deoxyribonucleic acid*
DR	receptor smrti *death receptor*
EAE	experimentální autoimunitní encefalomyelitida *experimental autoimmune encephalomyelitis*
EDAR	receptor proteinu ektodysplazin A *ectodysplasin A receptor*
Erk	kináza regulovaná extracelulárními signály *extracellular signal-regulated kinase*
FADD	protein asociovaný s receptorem Fas a obsahující doménu smrti *Fas-associated protein with death domain*
FLIP	protein inhibující kaspázu 8 *FLICE/CASP8 inhibitory protein*
GAP	protein aktivující GTPázy *GTPase-activating protein*
GFD	doména podobná růstovým faktorům *growth factor-like domain*
GITR	protein indukovaný glukokortikoidy a příbuzný receptoru TNFR *glucocorticoid-induced TNFR-related protein*
GM-CSF	faktor stimulující růst granulocytů a makrofágů *granulocyte macrophage colony-stimulating factor*
GPI	glykofosfatidylinositolová kotva *glycophosphatidylinositol anchor*
GVHD	reakce štěpu proti hostiteli *graft-versus-host disease*
HEK293	lidské zárodečné buňky ledvin, klon 293 *human embryonic kidney cells, clone 293*

HOIL	ubiquitin ligáza proteinu IRP2, oxidovaná hemem *heme-oxidized IRP2 ubiquitin ligase*
HOIP	protein interagující s proteinem HOIL-1 *HOIL-1-interacting protein*
HVEM	protein zprostředkovávající vniknutí herpesvirů do buňky *herpesvirus entry mediator*
ICAD	inhibitor DNázy aktivované kaspázami *inhibitor of caspase-activated DNase*
ICP	intracelulární část *intracellular part*
IFN	interferon
IgG	imunoglobulin G
IgM	imunoglobulin M
IKK	kináza faktoru IκB *IκB kinase*
IL	interleukin
JNK	kináza faktoru c-Jun *c-Jun N-terminal kinase*
LARD	receptor smrti asociovaný s lymfocyty *lymphocyte-associated receptor of death*
LPS	bakteriální lipopolysacharid
LT	lymfotoxin
LUBAC	komplex sestavující lineární řetězec proteinu ubiquitin *linear ubiquitin chain assembly complex*
MAPK	mitogeny aktivovaná proteinkináza *mitogen-activated protein kinase*
MCF7	buněčná linie karcinomu prsu *cell line "Michigan Cancer Foundation - 7"*
MMP	metaloproteáza *matrix metalloproteinase*
MOG	myelin oligodendrocyte glycoprotein
mRNA	mediátorová RNA *messenger RNA*
NCBI	National Center for Biotechnology Information
NEMO	protein nezbytný pro modulaci funkce faktoru NF-κB *NF-κB essential modulator*
NF-AT	transkripční faktor aktivovaných T-lymfocytů *nuclear factor of activated T-cells*

NF-κB	transkripční faktor zesilující expresi lehkého řetězce-κ v B-lymfocytech *nuclear factor of κ-light chain gene enhancer in B cells*
NGFR	receptor pro nervový růstový faktor *nerve growth factor-receptor*
NK	"*natural killer*" buňka
OCIF	faktor inhibující vývoj kostních buněk *osteoclastogenesis inhibitory factor*
OPG	osteoprotegerin
PARP	poly(ADP-ribóza)-polymeráza *poly(ADP-ribose)-polymerase*
PH	plecstrin homologní doména *plecstrin homology domain*
PHA	phytohemagglutinin
PI3K	fosfatidylinositol-3-kináza *phosphatidylinositol-3-kinase*
PKB	proteinkináza B *protein kinase B*
PLAD	doména umožňující sestavení komplexu s ligandem *pre-ligand assembly domain*
PMA	phorbol 12-myristate 13-acetate
PTEN	fosfatáza a homolog proteinu tensin *phosphatase and tensin homolog*
RAIDD	protein asociovaný s kinázou RIP, homologní kaspáze-2 a obsahující doménu smrti *RIP-associated ICH1/CASP2-homologous protein with death domain*
RANK	receptor aktivující faktor NF-κB *receptor activator of NF-κB*
RIP	protein-kináza interagující s receptory *receptor-interacting protein kinase*
RNA	ribonukleová kyselina *ribonucleic acid*
RT-PCR	reverzní transkripce - polymerázová řetezová reakce *reverse transcription - polymerase chain reaction*
SDS	dodecylsulfát sodný *sodium dodecyl sulfate*
siRNA	malá interferující RNA (krátká interferující RNA, umlčující RNA) *small interfering RNA (short interfering RNA, silencing RNA)*
Smac	druhý aktivátor kaspáz odvozený od mitochondrií *second mitochondria-derived activator of caspase*
TAB	protein vázající kinázu TAK1 *TAK1-binding protein*

TACE	enzym konvertující faktor TNFα *TNFα converting enzyme*
TACI	transmembránový protein aktivující a interagující s proteinem CAML *transmembrane activator and CAML interactor*
TAK	protein-kináza aktivovaná faktorem TGFβ *TGFβ activated kinase*
TCR	antigenně specifický receptor T-lymfocytů *T-cell receptor*
TL1	molekula příbuzná ligandu TNF *TNF ligand-related molecule-1*
TNF	faktor nekrotizující nádory *tumor necrosis factor*
TNFR	receptor faktoru nekrotizujícího nádory *tumor necrosis factor-receptor*
TNFsf	rodina proteinů příbuzných ligandu TNF *TNF superfamily*
TNFRsf	rodina proteinů příbuzných receptoru TNFR *TNFR superfamily*
TRADD	protein asociovaný s receptorem TNFR1 a obsahující doménu smrti *TNFR1-associated death domain protein*
TRAF	faktor asociovaný s receptorem TNFR1 *TNFR1-associated factor*
TRAIL	ligand příbuzný faktoru TNF a indukující apoptózu *TNF-related apoptosis-inducing ligand*
TRAPS	syndrom periodických horeček asociovaný s receptorem TNFR1 *TNFR1-associated periodic fever syndrome*
TRID	receptor ligandu TRAIL bez intracelulární domény *TRAIL-receptor without an intracellular domain*
TRUNDD	receptor ligandu TRAIL se zkrácenou doménou smrti *TRAIL-receptor with a truncated death domain*
TWEAK	protein příbuzný faktoru TNF a slabě indukující apoptózu *TNF-related weak inducer of apoptosis*
VEGI	inhibitor růstu cévních endoteliálních buněk *vascular endothelial growth inhibitor*
XEDAR	receptor proteinu ektodysplazin A vázaný na chromozóm X *X-linked ectodysplasin A receptor*
XIAP	inhibitor apoptózy vázaný na chromozóm X *X-linked inhibitor of apoptosis*
Y2H	kvasinkový dvouhybridní systém *yeast-two-hybrid system*

I. Úvod a cíle práce

Proteiny z rodiny receptorů TNFR hrají významnou roli v mnoha biologických procesech, jako jsou buněčná proliferace, diferenciace, programovaná buněčná smrt, regulace imunitní odpovědi a hematopoezy, atd. Také se ale účastní vzniku a rozvoje některých chorob, např. autoimunitních onemocnění, neurodegenerativnívh chorob nebo potíží spojených s odhojováním transplantátů. Antagonisté faktoru TNF (např. *adalimumab*, *infliximab* či *etanercept*) již byly schváleny a jsou používány v humánní medicíně k léčení některých autoimunitních chorob. V současné době jsou ve vývoji i terapeutické nástroje založené na blokování či aktivaci funkce dalších členů rodiny receptorů TNFR, ve fázi klinického testování jsou např. farmakologické nástroje indukující apoptózu nádorových buněk závislou na receptorech ligandu TRAIL.

Receptor DR6 byl objeven v roce 1998. V následujících letech studie DR6-deficientních myší prokázaly jeho roli v regulaci imunitní odpovědi a naznačily možné využití terapeutických nástrojů založených na stimulaci či blokování funkce tohoto receptoru v humánní medicíně. Nicméně o struktuře proteinu DR6, regulaci jeho exprese, ligandech a dalších interagujících proteinech, signalizaci a funkci obecně (zvláště na lidském modelu) máme doposud jen velmi málo relevantních informací. Zevrubná molekulární a funkční charakterizace tohoto proteinu je přitom jednou z nezbytných podmínek pro jeho případné praktické využití. Hlavním cílem naší práce tedy bylo objasnění vlastností a funkcí receptoru DR6 na molekulární úrovni, a to zejména na lidských buňkách. Jednotlivé dílčí cíle této práce potom byly následující:

1. Molekulárně charakterizovat lidský DR6 včetně jeho posttranslačních modifikací a popsat funkci těchto modifikací.
2. Přispět k objasnění mechanizmů regulace exprese DR6 na lidských hematopoietických buňkách.
3. Identifikovat ligand(y) receptoru DR6, případně připravit agonistické monoklonální protilátky, a využít je ke studiu buněčné signalizace spojené s receptorem DR6.
4. Objevit nové proteiny interagující s cytoplazmatickou částí receptoru DR6 a objasnit funkci těchto proteinů v signalizaci spojené s receptorem DR6.

II.A. Abstrakt

Receptor DR6 ("receptor smrti-6", TNFRsf21/CD358) je členem rodiny TNFR, který se pravěpodobně účastní regulace proliferace a diferenciace T- a B-lymfocytů a neurálních buněk. Lidský DR6 je 655 aminokyselin dlouhý transmembránový protein typu I, který obsahuje čtyři domény CRD ve své extracelulární části a po jedné doméně DD a CARD v části cytoplazmatické. Nadprodukce DR6 v některých nádorových buňkách vede k apoptóze, a/nebo aktivaci transkripčního faktoru NF-κB a kináz rodiny JNK.

V první naší práci jsme se věnovali charakterizaci molekulárních vlastností receptoru DR6 včetně analýzy jeho posttranslačních modifikací. Zjistili jsme, že DR6 je extenzivně modifikovaný protein a jeho posttranslační modifikace zahrnují *S*-palmitylaci a jak *N*-, tak i *O*-glykosylaci. Šest *N*-glykosylačních a jedno *S*-palmitylační místo jsme zamapovali do příslušných aminokyselinových zbytků. Spojovací část mezi doménami CRD v extracelulární části DR6 a jeho transmembránovou doménou, která obsahuje oblast bohatou na serin, threonin a prolin se seskupenými predikovanými *O*-glykosylačními místy, je nezbytná pro transport DR6 na plazmatickou membránu. *N*-glykosylace, ale překvapivě nikoli *S*-palmitylace, může hrát roli ve směřování DR6 do lipidových raftů.

V další části této práce jsme shrnuli výsledky studie analyzující regulaci exprese DR6 v lidských hematopoietických buňkách. Zjistili jsme, že DR6 není exprimován neaktivovanými T- a B-lymfocyty z lidské periferní krve, ale jeho exprese je významně zvýšena v aktivovaných jak CD4+, tak i CD8+ T-lymfocytech po stimulaci TCR, a že tento nárůst exprese DR6 je závislý na transkripčních faktorech NF-κB a NF-AT. Narozdíl od primárních lymfocytů exprimují buňky lidské T-lymfoblastické leukemie Jurkat receptor DR6 již před stimulací, pravděpodobně díky konstitutivně aktivní signalizaci z kinázy PI3K, a po stimulaci je tato exprese DR6 významně potlačena zřejmě na úrovni transkripce. Dále jsme analyzovali vliv jednotlivých predikovaných vazebných míst transkripčních faktorů NF-κB a NF-AT v DR6 promotoru na expresi DR6.

Naše zjištění by tedy měla přispět jak k molekulární charakterizaci DR6, tak i k objasnění jeho role v imunitním systému.

II.B. Abstract

Death receptor-6 (DR6/TNFRsf21/CD358) is a receptor from the TNFR superfamily that likely participates in the regulation of proliferation and differentiation of T- and B-lymphocytes and neural cells. The 655-amino acid human DR6 is a type I transmembrane protein containing four cysteine-rich domains in its extracelular part and a death domain followed by the CARD-like region in its cytoplasmic part. Overexpression of DR6 in some cell lines leads to apoptosis, and/or to activation of nuclear factor NF-κB and stress kinases of the JNK family.

In the first part of our work we focused on molecular characterization of DR6, including the analysis of its posttranslational modifications. We found that DR6 is an extensively posttranslationally modified protein including *S*-palmitoylation and both *N*- and *O*-glycosylation. Six *N*-glycosylation and one *S*-palmitoylation sites were precisely mapped to appropriate asparagines and cysteine respectively. The juxtaposed linker region (between cystein-rich domains and the transmembrane part), which also contains Ser/Thr/Pro-rich region with clustered putative *O*-glycosylation sites, is required for the plasma membrane localization of DR6. *N*-glycosylation, but interestingly not *S*-palmitoylation, may play a role in targeting of DR6 into detergent-resistant glycosphingolipid-enriched microdomains.

In the next part of this work we cover our studies analyzing the regulation of DR6 expression in human hematopoietic cells. We found that DR6 is not expressed in resting peripheral blood T and B cells, but is significantly upregulated in activated both CD4+ and CD8+ T lymphocytes upon TCR-mediated stimulation in a NF-κB- and NF-AT-dependent manner. Unlike primary lymphocytes, Jurkat lymphoblastic leukemia T cells already express DR6 likely via constitutive activation of PI3K pathway and strongly downregulate DR6 expression upon stimulation via suppression of its transcription. Furthermore, we have analysed the role of predicted NF-κB- and NF-AT-binding sites in the DR6 promoter in the expression of DR6.

Studies and results presented in this thesis should contribute both to the molecular characterization of DR6 as well as to to the elucidation of the role of DR6 in the immune system.

III. Literární přehled

III.1. Proteiny rodiny TNFR

III.1.1. Historie

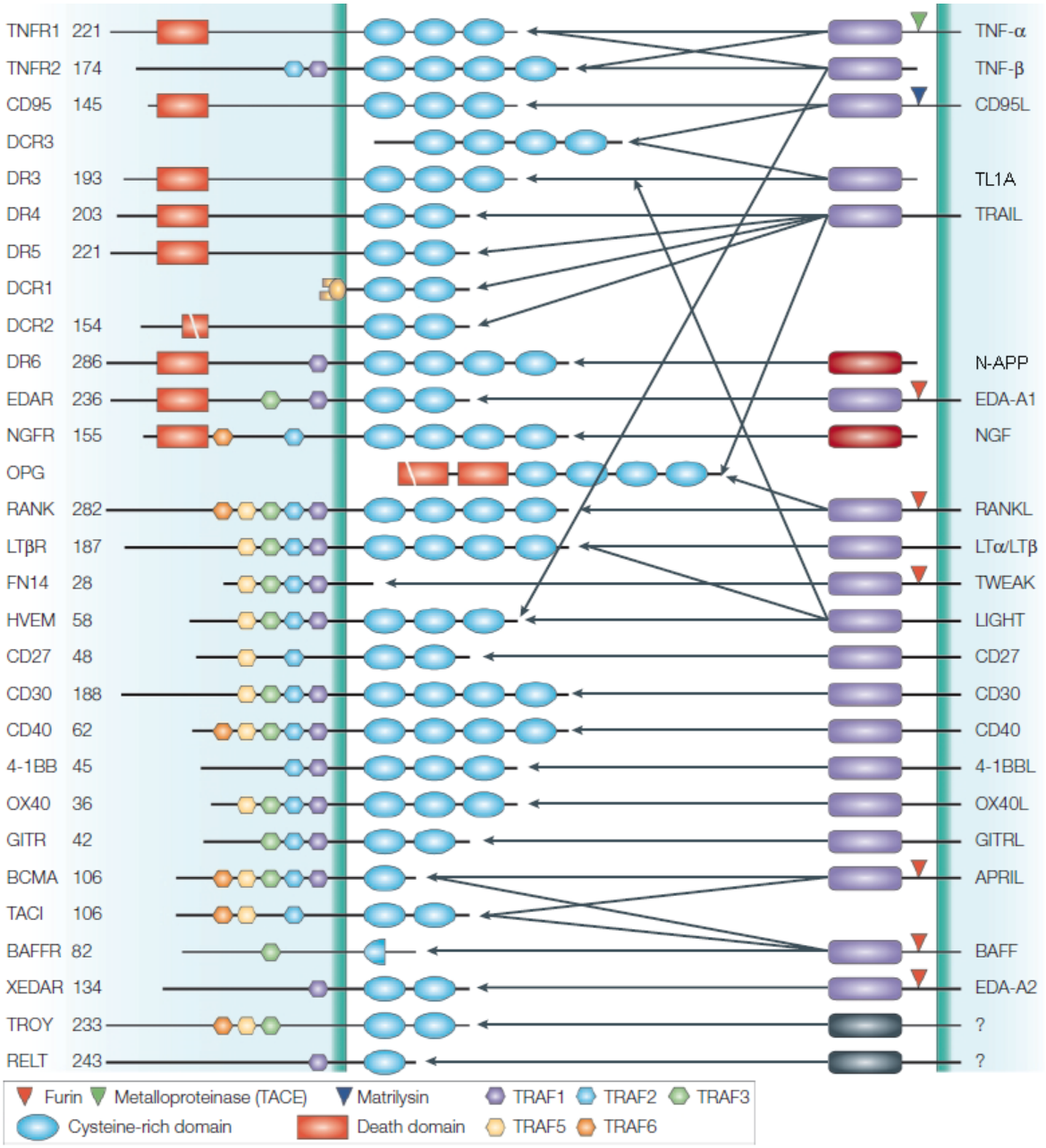
Receptory z rodiny TNFR a jejich ligandy, kterými jsou ve většině případů proteiny z rodiny TNF, začaly být postupně objevovány od 80. let 20. století, nicméně historie vědeckých objevů předcházejících izolaci prvního proteinu těchto rodin, faktoru nekrotizujícího nádory (TNFα), v roce 1984 je poměrně bohatá a sahá až do 19. století (Aggarwal, 2003). V roce 1868 německý lékař P. Bruns publikoval zjištění, že po bakteriální infekci dochází k regresi nádorů (Bruns, 1868). To později vedlo amerického onkologa a průkopníka nádorové imunoterapie W.B.Coleyho k používání bakteriálních extraktů k léčbě nádorů (Coley, 1891).

V roce 1943 byl poprvé z bakteriálních extraktů izolován bakteriální lipopolysacharid (LPS) jakožto původce pozorované regrese nádorů (Shear & Turner, 1943). Nicméně v roce 1963 bylo zjištěno, že tento efekt LPS není přímý, ale zprostředkovaný sérovým faktorem, nazvaným faktor nekrotizující nádory (TNF) (O'Malley et al., 1963). Jako buněčný zdroj tohoto faktoru byly později identifikovány aktivované makrofágy (Carswell et al., 1975). Aminokyselinová sekvence tohoto proteinu byla určena až po jeho purifikaci v roce 1984 (Aggarwal et al., 1985b; Aggarwal et al., 1984).

V dalších letech byly rychle objevovány další proteiny příbuzné faktoru TNFα (ligandy rodiny TNF) a jejich receptory (receptory rodiny TNFR). V současné době je známo 29 receptorů rodiny TNFR a 19 ligandů rodiny TNF a zdá se, že tento počet je již konečný. Přehled receptorů rodiny TNFR a ligandů rodiny TNF viz obr. 1.

III.1.2. Struktura a rozdělení

Ligandy rodiny TNF jsou obvykle transmembránové proteiny II. třídy, s intracelulárním *N*-terminálním koncem. Výjimku představují sekretované proteiny TNFβ/LTα a TL1/VEGI. Ostatní proteiny se v mnoha případech mohou kromě formy vázané na buněčnou membránu vyskytovat též v solubilní podobě po odštěpení své extracelulární části, nejčastěji membránovými metaloproteázami aktivovanými různými stimuly. Dobře popsanými případy jsou například odštěpování ligandu TNFα metaloproteázou ADAM17/TACE (Black et al., 1997) nebo ligandu FasL proteázou MMP7/matrilysin (Powell et al., 1999).



Obr. 1:

Receptory rodiny TNFR a jejich ligandy

Receptory rodiny TNFR (v levé části panelu) jsou obvykle transmembránové proteiny I. třídy, obsahují tzv. domény CRD ("*cystein-rich domain*") v extracelulární části. Některé z nich, tzv. receptory smrti ("*death receptors*") obsahují tzv. doménu DD ("*death domain*") v intracelulární části.

Jejich ligandy (v pravé části panelu) jsou obvykle proteiny rodiny TNF (nepříbuzné jsou pouze ligandy NGF a *N*-APP). Jsou to obvykle transmembránové proteiny II. třídy, obsahují tzv. TNF-homologní doménu v extracelulární části.

Podrobnější popis viz dále v textu.

Upraveno dle *Aggarwal, Nature Reviews in Immunology, 2003.*

Ligandy rodiny TNF jsou obvykle molekuly tvaru kompaktního "soudečku" tvořeného převážně β-strukturami, antiparalelními úseky polypeptidového řetězce. Biologicky aktivní jsou ve formě samovolně se ustanovujících, nekovalentně vázaných homotrimerů. Extracelulární *C*-terminální část, zvaná TNF-homologní doména, obsahuje cca 20-30% aminokyselinových zbytků identických mezi jednotlivými členy rodiny TNF. Jedná se většinou o hydrofobní zbytky zajišťující trimerní terciární strukturu těchto proteinů, zatímco méně konzervované polární zbytky lokalizované na vnějším povrchu jsou zodpovědné za vazbu na příslušné receptory (Eck & Sprang, 1989).

Zcela nepříbuzné ostatním ligandům receptorů rodiny TNFR jsou proteiny NGF (ligand receptoru NGFR) a *N*-APP (ligand receptoru DR6), které vůbec nejsou členy rodiny TNF (Bothwell, 1996; Nikolaev et al., 2009).

Receptory rodiny TNFR jsou obvykle transmembránové proteiny I. třídy, s extracelulární *N*-terminální částí. Výjimku představují sekretované proteiny OPG a DcR3, a dále receptor TRAIL-R3/DcR1, který je s membránou asociovaný pouze pomocí GPI-kotvy. Receptory BCMA, TACI, BAFFR a XEDAR postrádají signální peptid, a proto bývají formálně řazeny mezi transmembránové proteiny III. třídy. Podobně jako v případě ligandů rodiny TNF i některé receptory rodiny TNFR mohou být ve své extracelulární části odštěpovány, nejčastěji opět membránovými metaloproteázami. Solubilní forma receptorů Fas a 4-1BB vzniká pomocí alternativního sestřihu (Aggarwal, 2003).

Společným znakem všech receptorů rodiny TNFR je přítomnost tzv. domén CRD ("*cystein-rich domains*", domény bohaté na cystein) v jejich extracelulárních částech. Tyto domény jsou obvykle dlouhé cca 40 aminokyselin a každá z nich obsahuje šest velmi konzervovaných cysteinových zbytků vytvářejících tři intramolekulární disulfidické můstky. Počet domén CRD v molekule se pohybuje od jedné neúplné domény CRD u receptoru BAFFR až po šest domén CRD u receptoru CD30. Domény CRD jsou zodpovědné za interakci receptorů z rodiny TNFR s jejich příslušnými ligandy, obvyklá stechiometrie je 3:3, tzn. že trimerní ligand vytváří komplex se třemi molekulami příslušného receptoru. 3D struktura komplexu ligandu s receptorem na základě rentgenové strukturní krystalografie je známa u komplexu receptoru TNFR1 s ligandem TNFβ/LTα (Banner et al., 1993), dále u komplexů DR5/TRAIL (Hymowitz et al., 1999), NGFR/NGF (Gong et al., 2008), RANK/RANKL (Liu et al., 2010), DcR3/TL1A (Zhan et al., 2011) a CD40/CD40L.

Některé receptory rodiny TNFR (např. Fas, TNFR1, TNFR2) mohou vytvářet trimerní struktury i bez přítomnosti jejich ligandů. Za tento jev je zodpovědná jejich *N*-koncová oblast zahrnující část první domény CRD, tzv. doména PLAD ("*preligand assembly domain*"). Trimerní struktury

receptorů závislé na doméně PLAD jsou odlišné od struktur závislých na vazbě ligandu, nicméně jsou nezbytným předpokladem pro vazbu ligandu a následný přenos signálu (Chan et al., 2000).

Cytoplazmatické části receptorů rodiny TNFR mají proměnlivou délku a slouží k vazbě dalších molekul účastnících se přenosu signálu po spuštění buněčné signalizace příslušnými ligandy. Nejdůležitější adaptérové proteiny interagující s intracelulárními částmi receptorů rodiny TNFR patří do dvou skupin:

(1) adaptérové proteiny skupiny **TRAF** ("*TNF receptor-associated factors*", faktory asociované s receptorem pro TNF), obvykle zprostředkovávají buněčnou signalizaci vedoucí k aktivaci transkripčního faktoru NF-κB a mitogeny aktivovaných proteinkináz (MAPK) jako JNK1/2, Erk1/2 a p38 (Bradley & Pober, 2001; Lee & Lee, 2002). Nejlépe prostudovaným modelem v tomto směru je buněčná signalizace z receptoru TNFR1, o níž bude blíže pojednáno v kapitole III.2.2.2. (viz str. 23).
(2) adaptérové proteiny **FADD** a **TRADD** obsahující doménu DD ("*death domain*", doména smrti), obvykle zprostředkovávají buněčnou signalizaci vedoucí k aktivaci kaspázové kaskády a ke spuštění programované buněčné smrti (Park et al., 2007). V tomto směru je nejlépe prostudována buněčná signalizace z receptoru Fas, o níž bude blíže pojednáno v kapitole III.2.1.2. (viz str. 20).

S adaptérovými proteiny obsahujícími doménu DD interaguje jen malá podskupina receptorů rodiny TNFR, tzv. receptory smrti ("*death receptors*"), které samy obsahují doménu DD ve svých intracelulárních částech. Do této skupiny je řazeno celkem 8 proteinů, a to receptory Fas, TNFR1, DR3, DR4/TRAIL-R1, DR5/TRAIL-R2, DR6, NGFR a EDAR (Ashkenazi & Dixit, 1998; Wajant, 2003). Jelikož hlavní objekt zájmu této práce, receptor DR6, je členem této skupiny proteinů, je o ní podrobněji pojednáno v kapitole III.2. (viz str. 19).

III.1.3. Funkce a význam proteinů rodiny TNFR

Za fyziologických podmínek jsou receptory rodiny TNFR a jejich ligandy důležitými molekulami ve zprostředkovávání mnoha důležitých funkcí v organizmu, jako jsou buněčná proliferace, diferenciace, programovaná buněčná smrt, regulace imunitní odpovědi a hematopoezy, imunitní dozor a ochrana před vznikem nádorů. Naproti tomu špatná regulace exprese nebo funkce těchto proteinů může být pro organizmus značně poškozující a může být příčinou vzniku a rozvoje mnoha onemocnění (Aggarwal, 2003).

Receptor TNFR1 a jeho ligand TNFα je důležitý pro fungování vrozené, neadaptivní imunity a pro ochranu proti intracelulárním parazitům, viz kapitolu III.2.2.1. (str. 22). Některé receptory rodiny TNFR fungují jako kostimulační molekuly T-lymfocytů a jejich ligandy slouží po prvotní aktivaci T-buněk k udržení T-buněčné odpovědi, mezi tyto proteiny patří zejména CD27, CD30, OX40, 4-1BB/CD137, HVEM/LIGHTR a GITR (Watts, 2005). Receptor CD40 funguje jako kostimulační molekula B-lymfocytů, je nezbytný pro jejich diferenciaci a proliferaci, pro izotypový přesmyk a vznik germinálních center (Elgueta et al., 2009).

Pro programovanou buněčnou smrt mají význam především proteiny ze skupiny "receptorů smrti", zejména Fas/CD95, TNFR1, DR4/TRAIL-R1 a DR5/TRAIL-R2 (Ashkenazi & Dixit, 1998; Wajant, 2003), viz kapitolu III.2. (str. 19). Mezi jejich hlavní funkce patří aktivace apoptózy infikovaných buněk, lymfocytů na konci buněčné odpovědi a případně buněk nádorových. Jejich funkci mohou negativně regulovat tlumivé receptory DcR1/TRAIL-R3, DcR2/TRAIL-R4, DcR3 a OPG (Ashkenazi & Dixit, 1999). Nejvýznamnějším ligandem rodiny TNF pro imunitní dozor a ochranu před vznikem nádorů je zřejmě ligand TRAIL, který se v současné době jeví také jako nadějný cíl farmakologického výzkumu pro vývoj nových protinádorových terapeutik (Ashkenazi et al., 2008), viz kapitolu III.2.4. (str. 27).

Hlavní funkce některých dalších členů rodiny TNFR a jejich ligandů má těžiště mimo imunitní tkáň, např. receptory EDAR a XEDAR mají význam především pro vývoj kůže a kožních derivátů (Cui & Schlessinger, 2006), receptor NGFR pro vývoj nervové tkáně (Dechant & Barde, 2002) anebo receptor RANK pro vývoj kostní tkáně a mléčných žláz (Theill et al., 2002).

Je známo několik případů, kdy jsou konkrétní mutace v genech kódujících některé receptory rodiny TNFR nebo jejich ligandy spojeny se vznikem a rozvojem specifických onemocnění, např.

- syndrom hyperimunoglobulinémie IgM - mutace v genech pro CD40 nebo CD40L (Allen et al., 1993; Ferrari et al., 2001)
- autoimunitní lymfoproliferativní syndrom (ALPS) - mutace v genech pro Fas nebo FasL (Fisher et al., 1995; Wu et al., 1996)
- ektodermální dysplázie - mutace v genech pro EDAR nebo EDA1 (Monreal et al., 1999; Zonana et al., 1993)
- syndrom periodických horeček asociovaný s receptorem TNFR1 (TRAPS) (McDermott et al., 1999)

Častější jsou onemocnění spojená se špatnou regulací exprese či funkce receptorů rodiny TNFR nebo jejich ligandů, a to obvykle s jejich nadprodukcí či zvýšenou signalizací. Ta může vést k zánětlivým autoimunitním onemocněním, jako jsou revmatoidní arthritida (Nadkarni et al., 2007),

Bechtěrevova choroba (Gorman et al., 2002), Crohnova choroba a ulcerózní kolitida (Stokkers et al., 1995), psoriáza (Leonardi et al., 2003) a další. Proteiny rodin TNFR a TNF jsou však spojovány i s celou řadou dalších chorob, jako jsou neurodegenerativní onemocnění (Alzheimerova a Huntingtonova choroba), atheroskleróza, odhojování transplantátů, ischemické choroby, toxiny-indukované choroby jater, apod.

III.2. Proteiny skupiny "receptorů smrti"

III.2.1. Fas

III.2.1.1. Receptor Fas a jeho funkce

Receptor Fas (TNFRsf6, CD95, Apo1) byl původně objeven a v roce 1991 klonován jako antigen monoklonálních protilátek schopných spouštět buněčnou smrt (Itoh et al., 1991). Jeho fyziologický ligand FasL (TNFsf6, CD178, Apo1L) byl identifikován roku 1993 (Suda et al., 1993). Zatímco receptor Fas je produkován v buňkách mnoha tkání, exprese jeho ligandu FasL je omezena zejména na aktivované T-lymfocyty, NK-buňky a buňky imunoprivilegovaných tkání. Receptor Fas a jeho ligand FasL hrají důležitou roli v imunitním systému, mezi jejich hlavní funkce patří účast na zabíjení buněk infikovaných patogeny, odstraňování již nepotřebných a potenciálně nebezpečných lymfocytů na konci imunitní odpovědi a zabíjení zánětlivých buněk v imunoprivilegovaných tkáních.

Spontánně se vyskytující mutace v genech pro Fas a FasL v myších kmenech *lpr* a *gld* vedou k autoimunitním chorobám podobným nemoci systémový lupus erythematosus (Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992). Mutaci v genu pro Fas má i významné procento lidských pacientů trpících chorobou autoimunitní lymfoproliferativní syndrom (Fisher et al., 1995; Rieux-Laucat et al., 1995). Tito pacienti mají též zvýšenou predispozici k vývoji lymfomů, což dokládá význam proteinů Fas a FasL pro imunitní dozor a potlačování vývoje nádorů, minimálně v lymfatické tkáni (Straus et al., 2001). Farmakologické nástroje modulující funkci proteinů Fas a FasL by teoreticky mohly být využity v humánní medicíně pro léčbu těchto chorob, nicméně dosud velkým praktickým problémem je extensivní apoptóza hepatocytů vedoucí k fatální hepatitidě, kterou způsobují jak agonistické protilátky proti receptoru Fas (Ogasawara et al., 1993), tak užití rekombinantního ligandu FasL (Huang et al., 1999).

III.2.1.2. Buněčná signalizace z receptoru Fas, aktivace kaspázové kaskády

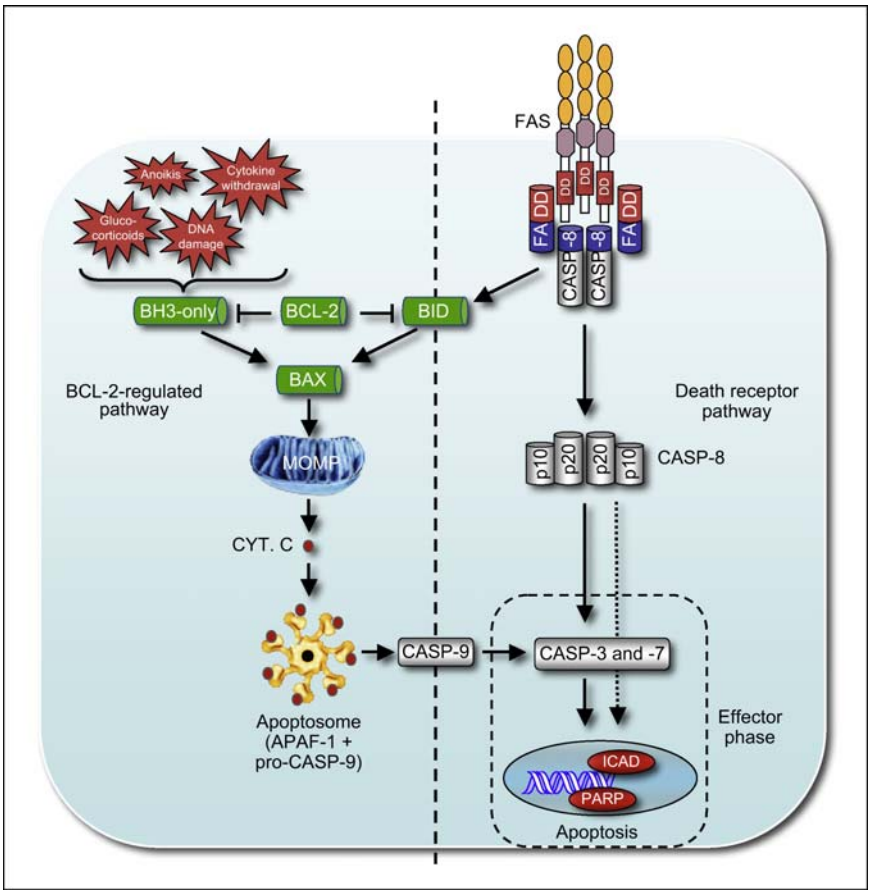
Velmi dobře prostudované jsou molekulární mechanizmy buněčné signalizace spouštěné vazbou ligandu FasL na receptor Fas. FasL je homotrimer, jehož vazba na receptor Fas iniciuje strukturní změny v jeho intracelulární části, umožňující sestavení komplexu DISC ("*death-inducing signaling complex*") (Kischkel et al., 1995). Ten sestává ze samotného receptoru Fas, dále z adaptérového proteinu FADD/Mort1 (Boldin et al., 1995; Chinnaiyan et al., 1995), regulačního proteinu c-FLIP (Irmler et al., 1997) a proteázy kaspáza-8/FLICE (Boldin et al., 1996; Muzio et al., 1996). Sestavení komplexu DISC je zprostředkované homotypickými interakcemi mezi doménami DD ("*death domain*") přítomnými v proteinech Fas a FADD, a mezi doménami DED ("*death effector domain*") přítomnými v proteinech FADD a kaspáza-8. V komplexu DISC dochází k dimerizaci a autoproteolytickému štěpení kaspázy-8, což vede k její plné enzymatické aktivitě. Aktivní kaspáza-8 následně opouští komplex DISC a může štěpit další substráty (Boatright et al., 2003; Oberst et al., 2009). V některých buněčných typech může kaspázu-8 funkčně nahradit příbuzná kaspáza-10 (Muhlethaler-Mottet et al., 2011; Vincenz & Dixit, 1997).

Protein c-FLIP je strukturně podobný kaspáze-8, ale nevykazuje enzymatickou aktivitu. Při nízké expresi stimuluje aktivaci kaspázy-8, pravděpodobně díky vyšší afinitě kaspázy-8 k proteinu c-FLIP než k sobě samé. Při vyšší expresi protein c-FLIP naopak aktivaci kaspázy-8 snižuje (a tím inhibuje apoptózu), pravděpodobně kompeticí s kaspázou-8 o vazbu na adaptérový protein FADD (Yeh et al., 2000; Zhang & He, 2005).

Různé buněčné typy se liší v mechanizmu, jakým dochází po aktivaci iniciátorových kaspáz (kaspázy-8 nebo -10) k aktivaci efektorových kaspáz (zejména kaspázy-3, -6 a -7) a k vlastní exekuci apoptózy. V buňkách typu I (např. lymfocyty) jsou efektorové kaspázy aktivovány přímo proteolytickým štěpením zprostředkovaným iniciátorovými kaspázami (tzv. "*extrinsic pathway*") (Kaufmann et al., 2007), zatímco v buňkách typu II (např. hepatocyty nebo pankreatické β-buňky) je kaspázová kaskáda amplifikována přes mitochondriální proteiny z rodiny Bcl-2 (tzv. "*intrinsic pathway*") (Kaufmann et al., 2009; McKenzie et al., 2008). Diskriminujícím faktorem mezi typem I a II aktivace kaspázové kaskády je míra aktivace kaspázy-8, případně úroveň exprese apoptotického inhibitoru XIAP (Deveraux et al., 1997; Jost et al., 2009) a jeho inhibitoru Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000).

Klíčovým proteinem regulujícím aktivaci kaspázové kaskády v buňkách typu II je protein Bid z rodiny Bcl-2. Je substrátem kaspázy-8 a jeho štěpená forma tBid ("*truncated Bid*") má po translokaci na vnější mitochondriální membránu proapoptotickou funkci (Li et al., 1998; Luo et al., 1998). Patří do skupiny proteinů obsahujících pouze doménu BH3 ("*BH3-only*"), společně

s proteiny Bad, Bik, Bim, Hrk, Noxa, Puma a Bmf (Huang & Strasser, 2000). Dalšími dvěma skupinami proteinů rodiny Bcl-2 jsou proapoptotické proteiny příbuzné proteinu Bax ("*Bax-like*"), kam patří Bax, Bak a Bok (Green & Kroemer, 2004), a anti-apoptotické proteiny příbuzné proteinu Bcl-2 ("*Bcl-2-like*"), mezi které patří Bcl-2, Bcl-xL, Bcl-w, A1 a Mcl-1 (Youle & Strasser, 2008). Proapoptotické proteiny skupiny Bax jsou schopny vytvořit supramolekulární kanály ve vnější mitochondriální membráně, což způsobuje její destabilizaci a permeabilizaci, která v konečném důsledku vede k uvolnění cytochromu c a dalších proapoptotických proteinů z mitochondriálního mezimembránového prostoru do cytoplazmy. Tento proces vzájemnými interakcemi aktivují proapoptotické proteiny jako tBid a inhibují anti-apoptotické proteiny skupiny Bcl-2.



Obr. 2:

Buněčná signalizace z receptoru Fas

Po vazbě ligandu FasL na receptor Fas dochází k sestavení tzv. komplexu DISC ("death-inducing signaling complex") a v něm k aktivaci iniciátorové kaspázy-8. Ta může aktivovat efektorové kaspázy buď přímo ("*extrinsic pathway*") anebo nepřímo přes aktivaci mitochondriálních proteinů rodiny Bcl-2, permeabilizaci vnější mitochondriální membrány (MOMP), uvolnění cytochromu c a sestavení komplexu zv. apoptozóm ("*intrinsic pathway*").

Podrobnější popis viz dále v textu.

Převzato ze *Strasser, Immunity, 2009.*

Cytochrom c po uvolnění do cytoplazmy vytváří společně s proteinem Apaf1 a kaspázou-9 komplex zvaný apoptozóm. V něm dochází k aktivaci iniciátorové kaspázy-9, která je proteolytickým štěpením schopná dále aktivovat efektorové kaspázy, zejména kaspázy-3, -6 a -7 (Li et al., 1997; Srinivasula et al., 1998).

Aktivované efektorové kaspázy pak štěpí své finální substráty, což v konečném důsledku vede k organizované a zánět nevyvolávající dezintegraci buňky. Mezi nejlépe dokumentované substráty efektorových kaspáz patří protein ICAD/DFF45, inhibitor DNázy CAD/DFF40, která se po aktivaci translokuje do buněčného jádra a štěpí jadernou DNA (Enari et al., 1998; Sakahira et al., 1998). Kaspázy také štěpí jaderné laminy, čímž dochází ke kondenzaci chromatinu a rozpadu buněčného jádra (Buendia et al., 1999; Rao et al., 1996), a některé proteiny účastnící se regulace stavby cytoskeletu, což vede v konečném stádiu k dezintegraci buňky na apoptotická tělíska (Kothakota et al., 1997).

III.2.2. TNFR1

III.2.2.1. Receptor TNFR1 a jeho funkce

Receptor TNFR1 ("*tumor necrosis factor-receptor 1*", TNFRsf1A, CD120a, p55/p60) je receptorem pro dva ligandy z rodiny TNF (Aggarwal et al., 1985a)

- **TNFα** (TNFsf2, cachectin) (Aggarwal et al., 1985b)
- **LTα** (TNFsf1, TNFβ, lymphotoxin) (Aggarwal et al., 1984)

Tyto proteiny se staly v letech 1984-5 prvními identifikovanými ligandy z rodiny TNF, nicméně historie vědeckých objevů předcházejících jejich izolaci sahá až do 19. století, viz kapitolu III.1.1. (str. 14).

Zatímco receptor TNFR1 je konstitutivně exprimován buňkami většiny tkání, ligand TNFα je produkován především aktivovanými makrofágy, v malé míře pak dalšími buněčnými typy jako lymfocyty, mastocyty, endoteliálními buňkami a fibroblasty. Velká množství TNFα jsou uvolňována v reakci na bakteriální lipopolysacharid. TNFα vykazuje široké spektrum biologických aktivit, především je hlavním prozánětlivým cytokinem, je však i mediátorem buněčné proliferace a diferenciace, anebo programované buněčné smrti. Je také významným faktorem v rozvoji mnoha autoimunitních chorob, např. revmatoidní arthritidy, Crohnovy choroby, ulcerózní kolitidy, psoriázy a dalších. Některé blokátory TNFα dokonce byly schváleny a jsou používány v humánní medicíně k léčení těchto chorob. Patří mezi ně např. neutralizační monoklonální protilátky proti TNFα

adalimumab (Humira) a *infliximab* (Remicade), nebo *etanercept* (Enbrel), fúzní protein extracelulární části receptoru TNFR2 a lidského imunoglobulinu (Goldenberg, 1999; Mouser & Hyams, 1999; Rau, 2002).

Fenotyp TNFα-deficientních myší a TNFR1-deficientních myší je podobný, nicméně nepříliš výrazný (Bruce et al., 1996; Marino et al., 1997). Důvodem je pravděpodobně částečná funkční zastupitelnost proteinů TNFα a TNFR1 dalšími proteiny rodin TNF a TNFR . Delece genů pro TNFα nebo TNFR1 nemá vliv na životaschopnost myší či jejich schopnost reprodukce. Normální zůstává i jejich adaptivní, buněčně zprostředkovaná imunitní odpověď, závislá na protilátkách nebo CD8+ T-lymfocytech. Narušena je však jejich vrozená neadaptivní imunita, tyto myši jsou rezistentní vůči septickému šoku indukovaném bakteriálním LPS, jsou však také velmi citlivé na infekci intracelulárními parazity, např. *Mycobacterium tuberculosis* (Flynn et al., 1995), *Lysteria monocytogenes* (Rothe et al., 1994a) nebo *Leishmannia major* (Vieira et al., 1996). Snížená obranyschopnost vůči těmto parazitům je také nepříjemným vedlejším účinkem při léčbě některých autoimunitních chorob založené na blokování ligandu TNFα, viz výše (Wagner et al., 2002).

III.2.2.2. Buněčná signalizace z receptoru TNFR1, aktivace transkripčního faktoru NF-κB

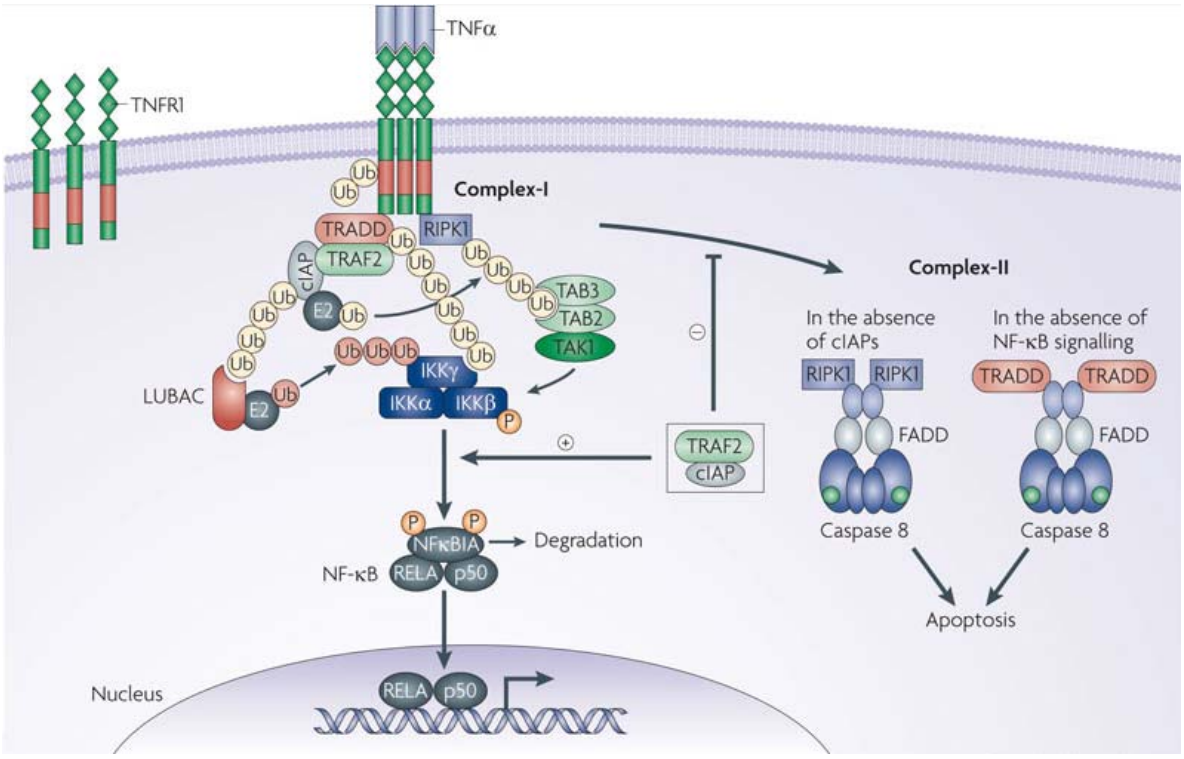
Buněčná signalizace spouštěná vazbou ligandu TNFα na receptor TNFR1 je velmi dobře prozkoumána a stala se vzorem při objasňování signálních drah využívaných dalšími receptory rodiny TNFR. Vazba ligandu TNFα na receptor TNFR1 iniciuje změny v jeho intracelulární části, umožňující sestavení receptorového komplexu zvaného komplex I (Micheau & Tschopp, 2003). Ten sestává ze samotného receptoru TNFR1, dále z adaptérového proteinu TRADD (Hsu et al., 1995), kinázy RIP1 (Hsu et al., 1996; Ting et al., 1996), ubiquitin-ligázy cIAP1 nebo cIAP2 (Rothe et al., 1995) a proteinu TRAF2 (Rothe et al., 1994b) nebo TRAF5 (Aizawa et al., 1997). Sestavení tohoto komplexu je umožněno homotypickými interakcemi mezi doménami DD přítomnými v proteinech TNFR1, RIP1 a TRADD, adaptérový protein TRADD pak slouží jako platforma pro vazbu ubiquitin-ligáz TRAF2/5 a cIAP1/2. V rámci tohoto komplexu pak dochází k poly-ubiquitinaci kinázy RIP1 (přes lysin K63) zprostředkované proteiny TRAF2/5 a cIAP1/2 (Bertrand et al., 2008; Mahoney et al., 2008; Varfolomeev et al., 2008). Tento proces je regulován enzymatickou aktivitou několika dalších proteinů, např. deubiquitinací kinázy RIP1 zprostředkovanou proteinem A20 (Wertz et al., 2004) nebo polyubiquitinací (přes lysin K48) kinázy RIP1 zprostředkovanou ubiquitin-ligázou CARP2 (Liao et al., 2008).

Ubiquitinace kinázy RIP1 přes lysin K63 neslouží jako její označení pro proteozomální degradaci (tak jako ubiquitinace přes lysin K48), ale jako platforma pro vazbu dalších tří proteinových komplexů:

(1) komplex kinázy **TAK1** (Wang et al., 2001) a TAK1-vazebných proteinů TAB1 a TAB2/3 (Kanayama et al., 2004)

(2) komplex **LUBAC** sestávající z ubiquitin-ligázy HOIL1 a HOIL1-interagujícího proteinu HOIP (Haas et al., 2009; Kirisako et al., 2006; Tokunaga et al., 2009)

(3) komplex **IKK** sestávající z protein-kináz IKKα a IKKβ a regulační podjednotky IKKγ/NEMO (Hacker & Karin, 2006)



Obr. 3:

Buněčná signalizace z receptoru TNFR1

Po vazbě ligandu TNFα na receptor TNFR1 dochází k sestavení tzv. komplexu I a v něm k ubiquitinaci několika proteinů (přes lysin K63) a k aktivaci komplexu IKK, což vede k aktivaci transkripčního faktoru NF-κB. Po internalizaci receptorového komplexu může vzniknout tzv. komplex II, v němž dochází k aktivaci kaspázy-8, což může vést k aktivaci kaspázové kaskády a k programované buněčné smrti jako v případě buněčné signalizace z receptoru Fas.

Podrobnější popis viz dále v textu.

Převzato z *Gyrd-Hansen, Nature Reviews in Cancer, 2010.*

Pro aktivaci komplexu IKK, která je klíčová pro další přenos signálu, jsou nutné nejméně dvě jeho posttranslační modifikace, a to fosforylace podjednotky IKKβ zprostředkovaná kinázou TAK1 a tzv. lineární ubiquitinace jeho regulační podjednotky IKKγ/NEMO zprostředkovaná ubiquitin-ligázou HOIL1. Aktivní komplex IKK fosforyluje proteiny rodiny IκB (např. IκBα), což vede dále k jejich polyubiquitinaci (přes lysin K48), proteozomální degradaci a uvolnění proteinů rodiny NF-κB (v tzv. kanonické dráze nejčastěji heterodimer podjednotek p50 a p65/RelA). Transkripční faktor NF-κB je pak translokován z cytoplazmy do buněčného jádra, kde aktivuje transkripci celé řady genů, např. prozánětlivých cytokinů nebo antiapoptotických faktorů jako cIAP1/2 a cFLIP (Karin & Lin, 2002).

Za určitých podmínek může dojít po vazbě ligandu TNFα na receptor TNFR1 a následné internalizaci receptorového komplexu ke vzniku tzv. komplexu II (Micheau & Tschopp, 2003; Schutze et al., 2008). Ten obsahuje buď proteiny TRADD, FADD a kaspáza-8 (tzv. komplex IIA) anebo proteiny RIP1, FADD a kaspáza-8 (tzv. komplex IIB), viz obr. 3 (Wang et al., 2008). V obou případech dochází k aktivaci iniciátorové kaspázy-8, ta následně po autoproteolytickém štěpení opouští komplex II a aktivuje kaspázovou kaskádu vedoucí k aktivaci efektorových kaspáz a programované buněčné smrti. Podobně jako v případě buněčné signalizace z receptoru Fas aktivace efektorových kaspáz v závislosti na buněčném kontextu může (buňky typu II, "*intrinsic pathway*") a nemusí (buňky typu I, "ex*trinsic pathway*") být závislá na mitochondriálních proteinech rodiny Bcl-2, viz kapitolu III.2.1.2. (str. 20).

Nedávno byla popsána další signální dráha spojená s receptorem TNFR1 vedoucí k buněčné smrti, která je podobná nekróze a nezávislá na proteinech FADD a kaspáza-8. Významnou roli v ní hraje komplex adaptérového proteinu RIP1 s příbuznou kinázou RIP3, a dochází k ní např. v případě, že kaspáza-8 je geneticky inaktivovaná nebo chemicky inhibovaná (He et al., 2009; Zhang et al., 2009). Pro tento typ buněčné smrti je používán název nekroptóza (Holler et al., 2000).

K regulaci buněčné odpovědi a k rozhodnutí mezi signalizací vedoucí k aktivaci transkripčního faktoru NF-κB, která je obvykle spojena s buněčnou proliferací a diferenciací, a signalizací vedoucí ke spuštění apoptózy nebo nekroptózy dochází na několika úrovních. Této regulace se mimo jiné účastní následující proteiny:

- antiapoptotický protein **cFLIP** - jeho exprese je aktivována faktorem NF-κB, inhibuje aktivaci kaspázy-8 v komplexu II kompeticí o vazbu na adaptérový protein FADD (Wang et al., 2008)
- antiapoptotické proteiny **cIAP1/2** - jejich exprese je také aktivována faktorem NF-κB, jsou nezbytné pro aktivaci kinázy RIP1 v komplexu I (Bertrand et al., 2008; Mahoney et al., 2008; Varfolomeev et al., 2008)

- proapoptotická ubiquitin-ligáza **ITCH** - její exprese je aktivována stresovými signály zprostředkovanými kinázami JNK1/2, aktivuje proteozomální degradaci proteinu cFLIP (Chang et al., 2006)
- proapoptotický protein **Smac**/DIABLO - přímou interakcí inhibuje proteiny cIAP1/2 (Du et al., 2000; Verhagen et al., 2000)
- proapoptotická deubiquitináza **CYLD** - aktivuje deubiquitinaci proteinů nezbytnou pro aktivaci faktoru NF-κB, např. proteinů TRAF2, RIP1 a IKKγ/NEMO (Trompouki et al., 2003)

Dominantní odpovědí na vystavení buněk působení ligandu TNFα je aktivace faktoru NF-κB. Nicméně přísná regulace buněčné signalizace z receptoru TNFR1 je pro organizmus naprosto zásadní, což dokazuje mimo jiné skutečnost, že je u myší letální genetická inaktivace mnohých signalizačních a regulačních molekul, jako jsou např. c-FLIP (Yeh et al., 2000), RIP1 (Kelliher et al., 1998), FADD (Yeh et al., 1998; Zhang et al., 1998) nebo kaspáza-8 (Varfolomeev et al., 1998). Naopak slučitelná s životem je současná genetická inaktivace FADD a RIP1 (Zhang et al., 2011), anebo kaspázy-8 a RIP3 (Kaiser et al., 2011; Oberst et al., 2011), což naznačuje významnou regulační úlohu kaspázy-8 v nekroptóze během embryonálního vývoje.

III.2.3. DR3

Death receptor-3 ("receptor smrti-3", DR3, TNFRsf25, Apo3, LARD, TRAMP, WSL-1) byl nezávisle na sobě objeven několika skupinami v roce 1996 (Bodmer et al., 1997; Chinnaiyan et al., 1996; Kitson et al., 1996; Marsters et al., 1996; Screaton et al., 1997b). Je strukturně velmi podobný receptoru TNFR1, narozdíl od běžně exprimovaného TNFR1 je však exprese receptoru DR3 omezena především na aktivované T-lymfocyty.

Prvním popsaným ligandem receptoru DR3 byl protein TWEAK (TNFsf12, Apo3L) (Chicheportiche et al., 1997; Marsters et al., 1998). Posléze však bylo zjištěno, že ligand TWEAK může spouštět programovanou buněčnou smrt nezávisle na receptoru DR3, včetně apoptózy buněk z DR3-deficientních myší (Kaptein et al., 2000; Schneider et al., 1999). Nakonec se ukázalo, že receptorem pro ligand TWEAK zřejmě vůbec není DR3, ale receptor Fn14 (TNFRsf12A, TWEAKR), další protein z rodiny receptorů TNFR (Wiley et al., 2001). V souladu s tímto zjištěním došlo ke změně systematického označení receptoru DR3 z dřívějšího TNFRsf12 na dnes užívané TNFRsf25.

Jediným dnes známým ligandem receptoru DR3 je tak protein TL1A (TNFsf15) (Migone et al., 2002), což je delší varianta již dříve známého proteinu TL1/VEGI popsaného jako inhibitor

buněčného růstu endoteliálních buněk (Tan et al., 1997; Yue et al., 1999; Zhai et al., 1999). Narozdíl od proteinu TL1, který je exprimován zejména na endoteliálních buňkách, je ligand TL1A produkován zejména aktivovanými dendritickými buňkami. Po vazbě na receptor DR3 může spouštět v závislosti na buněčném kontextu podobné signální dráhy jako v případě receptoru TNFR1, tzn. aktivaci transkripčního faktoru NF-κB a stresových kináz z rodiny JNK, případně aktivaci kaspázové kaskády vedoucí k programované buněčné smrti (Migone et al., 2002).

Interakce mezi ligandem TL1A a receptorem DR3 tak zprostředkovává signál mezi aktivovanými dendritickými buňkami a T-lymfocyty. Hlavními subpopulacemi T-lymfocytů exprimujícími DR3 jsou buňky typu Th1 a zejména typu Th17 (Pappu et al., 2008), což je nedávno popsaná subpopulace prozánětlivých T-lymfocytů, které sekretují IL-17 (Weaver et al., 2006). Významnou roli pak tyto buňky zřejmě hrají v rozvoji některých autoimunitních chorob (Kikly et al., 2006; McKenzie et al., 2006).

Ligand TL1A se tak skrze receptor DR3 účastní regulace proliferace Th17 lymfocytů. DR3-deficientní myši jsou rezistentní k některým experimentálně vyvolaným autoimunitním onemocněním, včetně experimentální autoimunitní encephalomyelitidy, ovalbuminem vyvolaného alergického zánětu dýchacích cest (Meylan et al., 2008) nebo experimentální kolagenem nebo antigenem indukované arthritidy (Bull et al., 2008; Zhang et al., 2009). Sníženou citlivost k těmto chorobám vykazují i TL1A-deficientní myši, role ligandu TL1A byla též prokázána v onemocněních jako jsou experimentálně vyvolaná ileitida (zánět kyčelníku) nebo kolitida (zánět tlustého střeva) (Bamias et al., 2006).

Využití antagonistických protilátek proti ligandu TL1A nebo rekombinantního solubilního receptoru DR3 by tak mohlo vést k vývoji terapeutických nástrojů pro léčbu onemocnění jako je revmatoidní arthritida apod. (Bayry, 2010).

III.2.4. Receptory pro ligand TRAIL

Ligand TRAIL ("*TNF-related apoptosis-inducing ligand*", TNFsf10, Apo2L) je protein spouštějící apoptózu buněk mnoha nádorových buněčných linií (Pitti et al., 1996; Wiley et al., 1995). Váže se celkem na pět receptorů rodiny TNFR:

- **DR4** ("*death receptor-4*", TNFRsf10a, TRAIL-R1, Apo2) (Pan et al., 1997b)
- **DR5** ("*death receptor-5*", TNFRsf10b, TRAIL-R2, Killer, Trick2) (MacFarlane et al., 1997; Pan et al., 1997a; Screaton et al., 1997a; Sheridan et al., 1997; Schneider et al., 1997; Walczak et al., 1997; Wu et al., 1997)

- **DcR1** ("*decoy receptor-1*", TNFRsf10c, TRAIL-R3, TRID) (Degli-Esposti et al., 1997b)
- **DcR2** ("*decoy receptor-2*", TNFRsf10d, TRAIL-R4, TRUNDD) (Degli-Esposti et al., 1997a; Marsters et al., 1997; Pan et al., 1998b)
- **OPG** (osteoprotegerin, TNFRsf11b, OCIF) (Simonet et al., 1997; Tsuda et al., 1997)

Receptory DR4 a DR5 mohou po vazbě ligandu TRAIL interagovat skze svoje domény DD v intracelulární části s adaptérovým proteinem FADD, což vede k podobné buněčné signalizaci jako v případě receptoru Fas, tedy k aktivaci kaspázové kaskády vedoucí k apoptóze, viz kapitolu III.2.1.2. (str. 20). Po internalizaci receptorového komplexu může vzniknout cytoplazmatický komplex II obsahující též proteiny TRADD, TRAF2 a RIP1, což vede k podobné buněčné signalizaci jako v případě receptoru TNFR1, tj. k aktivaci transkripčního faktoru NF-κB a MAP kináz JNK1/2 a p38, viz kapitolu III.2.2.2. (str. 23).

Zbývající tři receptory ligandu TRAIL nejsou schopny přenášet apoptotický signál a účastní se tak regulace buněčné odpovědi na působení ligandu TRAIL kompeticí právě o ligand TRAIL. Receptor DcR1 je s plazmatickou membránou asociovaný pomocí GPI-kotvy a zcela tak postrádá cytoplazmatickou část. Receptor DcR2 sice je transmembránovým proteinem, ale jeho doména DD je zkrácená a dysfunkční. Osteoprotegerin je sekretovaný protein, jehož hlavní funkcí je inhibice vývoje osteoklastů a resorpce kostní tkáně blokováním interakce mezi receptorem RANK a ligandem RANKL (který kromě receptoru RANK váže též i OPG). Ligand TRAIL by tak mohl vazbou receptoru OPG ovlivňovat diferenciaci a funkci osteoklastů (Zauli et al., 2008), nicméně v tomto směru u TRAIL-deficientních myší nebyly pozorovány žádné patologické změny (Labrinidis et al., 2008).

Vzhledem ke skutečnosti, že (narozdíl od příbuzných ligandů FasL a TNFα) ligand TRAIL vyvolává programovanou buněčnou smrt především u nádorových buněk, zatímco k normálním buňkám je toxický minimálně, se ligand TRAIL v posledních cca 10 letech stal kandidátem na vývoj farmakologických nástrojů s protinádorovými účinky. Zvláště první studie vypadaly velmi slibně, jak na myších (Walczak et al., 1999), tak i na primátech rodu *Macaca fascicularis* (Ashkenazi et al., 1999). Později se však ukázalo, že ligand TRAIL může vyvolávat apoptózu též u normálních lidských hepatocytů, což je podobná komplikace jako v případě ligandu FasL, znemožňující jeho přímé použití v humánní medicíně (Jo et al., 2000). Pozorovaná hepatotoxicita ligandu TRAIL byla zpochybněna v následující studii (Lawrence et al., 2001), zřejmě v tomto směru hraje značnou roli design a způsob přípravy různých forem rekombinantního ligandu TRAIL či jeho mutantů. Vývoj optimalizovaných forem rekombinantního ligandu TRAIL nebo agonistických protilátek proti jeho receptorům je tak stále předmětem farmakologického výzkumu

(Ashkenazi et al., 2008). V různých fázích klinického testování jsou v současné době např. komerční produkty firmy Genentech zvané *dulanermin* (rekombinantní ligand TRAIL) a *drozitumab* (agonistická protilátka proti receptoru DR5).

Zatímco schopnost rekombinantního ligandu TRAIL spouštět apoptózu především nádorových buněk je velmi dobře prozkoumána, jeho fyziologická funkce (a fyziologická funkce jeho receptorů) v organizmu je poněkud nejasná. Narozdíl od člověka se u myší vyskytuje pouze jediný receptor pro ligand TRAIL. TRAIL receptor-deficientní myši jsou životaschopné, s normálním vývojem a schopné reprodukce. Bylo pozorováno, že tyto myši mají zvýšenou vrozenou imunitu spojenou s výraznější imunitní odpovědí na virovou infekci (Diehl et al., 2004). U experimentálně vyvolaných nádorů u nich dochází k nárůstu metastáz lymfomů a kožních karcinomů (Grosse-Wilde et al., 2008) i k rychlejšímu růstu větších nádorů v případě hepatokarcinomů (Finnberg et al., 2008).

Podobný fenotyp byl zjištěn i v případě TRAIL-deficientních myší. I tyto myši mají normální embryonální vývoj bez pozorovaných závažných poruch apoptózy . U těchto myší byla pozorována snížená cytotoxicita NK buněk a také zvýšená náchylnost ke vzniku nádorů a metastáz (Cretney et al., 2002). Tato data tedy naznačují možnou fyziologickou funkci ligandu TRAIL a jeho receptorů DR4 a DR5 jakožto tumor supresorů.

III.3. Receptor DR6

III.3.1. Struktura genu DR6

Gen lidského DR6 (synonyma TNFRSF21, CD358, BM-018, MGC31965) je lokalizován na chromozómu 6, lokus 6p21.1. Obsahuje 6 exonů, jediná známá sestřihová varianta obsahuje překládanou oblast délky 1968 párů bází. Sekvence mRNA v databázi NCBI má kód NM_014452.3.

Gen myšího DR6 (synonyma Tnfrsf21, TR7, R74815, AA959878) je lokalizován na chromozómu 17, lokus 17C. I tento gen obsahuje 6 exonů a jediná známá sestřihová varianta rovněž obsahuje překládanou oblast délky 1968 párů bází. Sekvence mRNA v databázi NCBI má kód NM_178589.3.

Překládané oblasti genů lidského a myšího DR6 vykazují homologii cca 87%. Obecně struktura DR6 je evolučně poměrně konzervovaná, orthology DR6 vykazující vysoký stupeň homologie k lidskému DR6 byly identifikovány v mnoha dalších obratlovcích, včetně zebřičky (*Danio rerio*), kuru (*Gallus gallus*), krysy (*Rattus rattus*) a šimpanze (*Pan troglodytes*).

III.3.2. Struktura proteinu DR6

Otevřený čtecí rámec genu lidského DR6 kóduje transmembránový protein typu I délky 655 aminokyselinových zbytků, proteinová sekvence v databázi NCBI má kód NP_055267.1 (Pan et al., 1998a).

Prvních 41 aminokyselin (Met1-Ala41) tvoří signální peptid směřující DR6 na plazmatickou membránu.Extracelulární část maturovaného proteinu (Gln42-Leu350) obsahuje čtyři tzv. domény bohaté na cystein ("*cystein rich domains*", CRD, Leu50-Cys211) charakteristické pro všechny proteiny rodiny TNFR. Sekvenčně nejpříbuznější jsou analogické domény proteinů OPG a TNFR2, sekvenční shoda na proteinové úrovni dosahuje 36%, resp. 42%. Za poslední doménou CRD následuje doména přilehlá k membráně ("*juxtamembrane domain*", Gly212-Leu350), která je v kontextu ostatních receptorů rodiny TNFR nezvykle dlouhá a obsahuje oblast bohatou na aminokyseliny serin, threonin a prolin ("*SPT-rich region*", Gly212-Thr254).

20 aminokyselin dlouhá transmembránová doména (Pro351-Ile370) je následována 285 aminokyselin dlouhou intracelulární částí (Arg371-Leu655). Cytoplazmatická část DR6 obsahuje sekvenční motiv zvaný doména smrti ("*death domain*", DD, Pro400-Gly496), která zařazuje receptor DR6 do skupiny receptorů smrti rodiny TNFR ("*death receptors*", DRs). Největší homologii vykazuje doména smrti proteinu DR6 k obdobné doméně proteinu TNFR1, a to cca 27%. Narozdíl od ostatních receptorů smrti, které obsahují domény smrti ve svých *C*-koncových oblastech, doména smrti proteinu DR6 je přilehlá k transmembránové části. *C*-koncová oblast proteinu DR6 obsahuje doménu, jejíž terciární struktura je podobná sekvenčnímu motivu zvanému kaspázu rekrutující doména ("*caspase recruitment domain*", CARD, Ser564-Leu655).

III.3.3. Funkce DR6

Nadprodukce proteinů skupiny receptorů smrti může vyvolat apoptózu i nezávisle na přítomnosti jejich příslušných ligandů. I v případě nadprodukce receptoru DR6 v buňkách některých nádorových linií (např. HeLa, buňky lidského cervikálního karcinomu) dochází ke spouštění programované buněčné smrti, přičemž tento děj je závislý na doméně DD receptoru DR6. Překvapivě nadprodukce DR6 v buňkách MCF7 (buňky lidského karcinomu prsu) apoptózu nespouští, ačkoliv jsou tyto buňky citlivé na nadprodukci receptoru DR4. Předpokládá se proto, že DR6 využívá odlišný mechanizmus buněčné signalizace než ostatní proteiny skupiny receptorů smrti.

Pro přenos apoptotického signálu tyto receptory obvykle využívají adaptérový protein FADD (např. Fas, viz kapitolu III.2.1.2., str. 20), anebo současně FADD a TRADD (např. TNFR1, viz

kapitolu III.2.2.2., str. 23). Receptor DR6 neinteraguje s proteinem FADD a pouze velmi slabě interaguje s proteinem TRADD. Neinteraguje ani s dalšími známými vazebnými partnery příbuzných receptorů, jako jsou proteiny RAIDD a RIP1.

Nadprodukce receptoru DR6 v buňkách HEK293 (lidské embryonální buňky ledvin) dále vede k aktivaci transkripčního faktoru NF-κB a stresových kináz JNK1/2. Zatímco aktivace faktoru NF-κB závisí na přítomnosti domény DD v intracelulární části receptoru DR6, aktivace kináz JNK1/2 absencí této domény není významně ovlivněna (Pan et al., 1998a).

III.3.4. Myší model

Většina poznatků o funkci receptoru DR6 pochází ze studia myší s inaktivovaným DR6, tzv. myších knock-outů. DR6-deficientní myši byly připraveny nezávisle ve dvou laboratořích, ve skupinách Vishvy M. Dixita (Genentech, San Francisco) a Songqing Na (Eli Lilly & Company, Indianapolis). Jejich studium vedlo v obou případech k podobným pozorováním. DR6-deficientní myši jsou životaschopné, schopné reprodukce a v jejích hlavních orgánech nebyly pozorovány žádné výrazné patologické změny.

Hlavní rozdíly mezi myšmi se standardním fenotypem ("*DR6 wild-type*") a myšmi DR6-deficientními (DR6-/-) byly oběma skupinami pozorovány v imunitním systému, zvláště po jeho stimulaci. V případě absence DR6 byla pozorována zvýšená proliferace T-lymfocytů po stimulaci jejich T-receptoru. Aktivované DR6-/- CD4+ T-lymfocyty měly zvýšenou expresi molekuly CD25 (α-podjednotka receptoru pro interleukin-2, aktivační marker T-lymfocytů) a byla pozorována jejich zvýšená proliferace po stimulaci interleukinem-2. Dále měly tyto buňky zvýšenou expresi molekuly CD28 (kostimulační receptor T-lymfocytů) a sníženou expresi molekuly CTLA-4 (inhibiční receptor T-lymfocytů). Aktivované DR6-/- CD4+ T-lymfocyty produkovaly zvýšené množství cytokinů spojených s imunitní odpovědí typu Th2 (např. IL-4, IL-5, IL-10, IL-13), zatímco produkce cytokinů spojených s imunitní odpovědí typu Th1 (např. IL-2, IFN-γ) nebyla významně ovlivněna. Receptor DR6 se tak jeví jako regulační molekula imunitní odpovědi zprostředkované CD4+ T-lymfocyty (Liu et al., 2001; Zhao et al., 2001).

Podobně jako T-lymfocyty, také B-lymfocyty izolované z DR6-deficientních myší vykazovaly zvýšenou proliferaci po stimulaci faktory jako jsou LPS nebo stimulační protilátky proti imunoglobulinu M nebo molekule CD40. Tyto buňky měly po aktivaci též zvýšenou expresi kostimulačních molekul CD80 a CD86 a byly efektivnějšími antigen-prezentujícími buňkami. Důsledkem těchto změn je pak zvýšená produkce protilátek po imunizaci DR6-deficientních myší (Schmidt et al., 2003).

Pro potvrzení hypotézy, že receptor DR6 je molekulou podílející se na regulaci imunitní odpovědi, bylo využito několika experimentálně běžně používaných modelů relevantních onemocnění. Prvním z nich byla akutní reakce štěpu proti hostiteli ("*graft-versus-host disease*", GVHD), která je výsledkem aktivace a expanze alloreaktivních dárcovských T-lymfocytů po transplantaci kostní dřeně. Bylo zjištěno, že přenos dárcovských T-lymfocytů z DR6-deficientních myší způsobuje v příjemcích rychlejší nástup symptomů reakce štěpu proti hostiteli, ztrátu hmotnosti, časnější patologické změny v některých orgánech a rychlejší úmrtnost, pravděpodobně v důsledku zvýšené aktivace a expanze DR6-/- T-lymfocytů. Předpokládá se tak, že identifikace ligandů receptoru DR6 nebo příprava agonistických protilátek by mohla vést k jejich terapeutickému užití v léčbě onemocnění zprostředkovaných T-lymfocyty (Liu et al., 2002).

Dalším experimentálně běžně užívaným zvířecím modelem T-buněčně zprostředkovaných onemocnění je experimentální autoimunitní encefalomyelitida (EAE) vyvolaná imunizací myší peptidem z glykoproteinu MOG ("*myelin oligodendrocyte glycoprotein*"), která vede k infiltraci centrální nervové soustavy autoreaktivními T-lymfocyty a následné demyelinizaci podobně jako u roztroušené sklerózy. Ukázalo se, že DR6-deficientní myši jsou k vyvolání této choroby prakticky rezistentní. Po imunizaci proteinem MOG u nich došlo ke vzniku výrazně menšího množství zánětlivých ložisek s minimální demyelinizací a k minimální infiltraci míchy mononukleárními buňkami včetně CD4+ T-lymfocytů. Jedním z důvodů těchto pozorování může být snížená exprese integrinu VLA-4 aktivovanými DR6-deficientními T-lymfocyty, molekuly kritické pro infiltraci T-lymfocytů do centrální nervové soustavy (Schmidt et al., 2005).

V myším modelu astmatu, alergické přecitlivělosti dýchacích cest, jsou myši nejdříve imunizovány a poté jejich plíce znovu vystavovány určitému alergenu, obvykle ovalbuminu. U DR6-deficientních myší byly příznaky takto vyvolaného astmatu významně zeslabeny, včetně sníženého množství eosinofilních granulocutů a nižší hladiny cytokinů IL-5 a IL-13 v bronchoalveolárním sekretu. V kontextu předchozích prací, které naopak popisovaly zvýšenou produkci Th2 cytokinů DR6-deficientními T-lymfocyty, bylo toto pozorování poněkud překvapivé. Vysvětlením může být, že hlavním mechanizmem rezistence DR6-deficientních myší vůči uvedeným experimentálně indukovaným chorobám není disbalance imunitní odpovědi typu Th1 a Th2, nýbrž defekt ve schopnosti imunitních buněk postrádajících DR6 infiltrovat místo, kde se vyskytuje příslušný antigen (např. centrální nervová soustava u EAE, bronchoalveolární systém u astmatu) (Venkataraman et al., 2006).

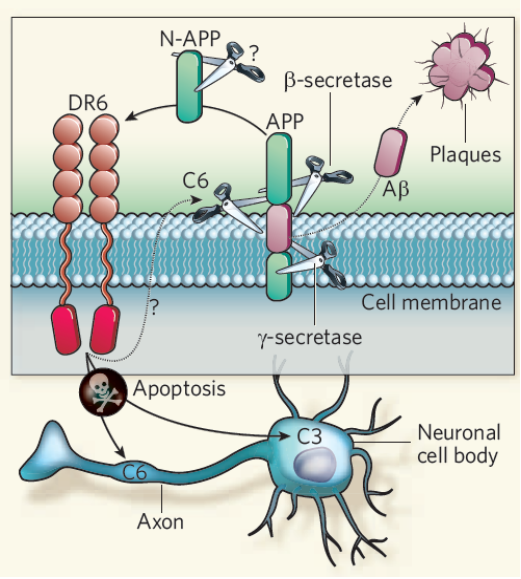
III.3.5. DR6 v nervovém systému

Zcela nový pohled na možnou fyziologickou funkci receptoru DR6 přinesla práce (Nikolaev et al.) publikovaná v roce 2009. Cílem této studie bylo přispět k objasnění molekulární podstaty spouštění buněčné smrti nervových buněk a degenerace neuronů, která probíhá přirozeně při vytváření nervových spojení během vývoje jedince, ale také patologicky v důsledku některých neurodegenerativních onemocnění, jako je Alzheimerova nebo Huntingtonova choroba.

Na modelu indukce programované buněčné smrti myších embryonálních míšních neuronů bylo ukázáno, že deprivace růstových faktorů spouští aktivaci proteáz β- a γ-sekretáza, které pak štěpí protein APP ("*β-amyloid precursor protein*") na několik fragmentů, a to *N*-koncovou solubilní část (*N*-APP), membránově vázaný amyloid-β (Aβ) a intracelulární doménu APP (AICD). Protein *N*-APP pak slouží jako ligand receptoru DR6 a může spouštět buněčnou signalizaci spojenou s DR6. Ta vede jednak k aktivaci kaspázy-3 v tělech neuronů a následně k buněčné smrti, a dále také k aktivaci kaspázy-6 v nervových vláknech vedoucí k degeneraci axonů. Tento proces je mimo jiné regulován též proteiny z rodiny Bcl-2, např. proteinem Bax.

Interakce receptoru DR6 s proteinem *N*-APP byla modelována *in silico*, s využitím známé krystalové struktury *N*-APP a počítačového homologního modelování DR6. Bylo zjištěno, že k interakci dochází mezi doménou GFD ("*growth factor-like domain*") proteinu *N*-APP a doménami CRD receptoru DR6 (Ponomarev & Audie, 2011).

Receptor DR6 tak může být důležitým faktorem pro vývoj nervového systému, ale může se zároveň také podílet na vývoji a progresi některých neurodegenerativních onemocnění (Nicholson, 2009).



Obr. 4:

Neurodegenerace závislá na buněčné signalizaci z receptoru DR6

Deprivace růstových faktorů vede k aktivaci proteáz β- a γ-sekretáza, které štěpí protein APP na fragmenty *N*-APP, Aβ a AICD. Protein *N*-APP pak spouští aktivaci kaspázové kaspády závislou na receptoru DR6, která vede k apoptóze neuronů či degeneraci axonů.

Podrobnější popis viz dále v textu.

Převzato z *Nicholson, Nature, 2009.*

III.3.6. DR6 jako ligand jiných receptorů

Pokud je ligandem transmembránový protein, může po interakci s jeho receptorem docházet k signalizaci nejen z intracelulární části receptoru, ale zároveň také z intracelulární části ligandu. Je pak jen umělým rozhodnutím, který z partnerů při této protein-proteinové interakci je označen jako receptor a který jako ligand. Vazba receptoru DR6 na jiný protein tak může také spustit buněčnou signalizaci z toho proteinu, vedoucí ke změně genové exprese v cílové buňce.

Receptor DR6 byl identifikován jako substrát proteázy MMP-14 ("*matrix metalloproteinase-14*"), enzymu asociovaného s membránou, který štěpí uvnitř extracelulární části DR6 (Tam et al., 2004). Odštěpená solubilní část DR6 by tedy skutečně mohla sloužit jako ligand jiného proteinu. Jediná dostupná data týkající se této problematiky byla publikována v práci (Derosa et al., 2007). Bylo zjištěno, že vystavení lidských primárních monocytů působení exogenního rekombinantního DR6 ovlivňovalo jejich *in vitro* diferenciaci do dendritických buněk pomocí IL4/GM-CSF. S tímto pozorováním však polemizujeme v naší práci (Klíma et al., 2009), zdá se, že popsaný efekt byl vyvolán spíše nespecificky a nezávisle na receptoru DR6 užitím nevhodně připraveného rekombinantního solubilního DR6. Fyziologická funkce solubilní části DR6 vzniklé odštěpením pomocí proteázy MMP-14 tak zůstává nadále neznámá.

IV. Výsledky a diskuse

IV.1. Funkční analýza posttranslačních modifikací receptoru DR6

(komentář k publikaci Klíma M., Zájedová J., Doubravská L., Anděra L.: Functional analysis of the posttranslational modifications of the death receptor 6. Biochim Biophys Acta, 2009)

Jednou z motivací pro naši studii vlastností receptoru DR6 byl zásadní rozpor mezi očekávanou (a dokonce i dříve uváděnou) a skutečnou mobilitou tohoto proteinu v polyakrylamidovém gelu během SDS elektroforézy, která odpovídá jeho zdánlivé molekulové hmotnosti. Molekulová hmotnost prekurzoru lidského DR6 vypočtená z jeho aminokyselinové sekvence je cca 72kDa, po odštěpení signálního peptidu pak cca 68kDa. Protilátky proti lidskému DR6 komerčně dostupné v době, kdy tato práce vznikala, skutečně rozlišovaly nějaký protein o zdánlivé molekulové hmotnosti kolem 68kDa v lyzátech většiny nádorových linií a molekulová hmotnost kolem 68kDa byla uváděná jako molekulová hmotnost lidského DR6 dokonce i v některých publikacích, např. (Pan et al., 1998a) a (Bridgham et al., 2001).

Králičí polyklonální protilátka proti DR6 připravená v naší laboratoři také rozeznávala nějaký protein o zdánlivé molekulové hmotnosti kolem 68kDa (dále značen jako p68) v lyzátech většiny nádorových linií. Nicméně první zásadní rozpor přineslo pozorování, že transfekce plasmidu pro eukaryotickou expresi lidského DR6 do buněk HEK293T vede k nadprodukci dvou proteinů o zdánlivých molekulových hmotnostech cca 110kDa a 85kDa (dále značeny jako p110 a p85). Následný experiment ukázal, že malá množství proteinů p110 a p85 jsou endogenně exprimována i buňkami některých lidských nádorových linií hematopoietického i nehematopoietického původu (např. nádorové linie Jurkat, KM3 a NCTC). Navazující důsledná analýza pak prokázala následující zjištění:

(1) Exprese forem p110 a p85 v buňkách různých lidských nádorových linií zjištěná pomocí imunoprecipitace našimi monoklonálními protilátkami proti DR6 a následnou imunodetekcí pomocí naší králiči polyklonální protilátky proti DR6 výborně korelovala s povrchovou expresí receptoru DR6 zjištěnou pomocí průtokové cytofluorometrie i s expresí mRNA zjištěnou pomocí metody kvantitativní RT-PCR. Naproti tomu protein p68 je exprimován většinou testovaných nádorových linií v přibližně stejné míře.
(2) Potlačení exprese DR6 pomocí siRNA v buňkách NCTC vedlo k výraznému snížení exprese forem p110 a p85, nikoli však ke změně exprese formy p68.

(3) Působením faktoru TNFα na buňky nádorové linie LnCAP dochází k nárůstu exprese forem p110 a p85, který koreluje s obdobným nárůstem množství transkriptu DR6 zjištěným pomocí kvantitativní RT-PCR. Naproti tomu exprese formy p68 se nemění.

(4) Frakcionace buněčných lyzátů ukázala, že formy p110 a p68 jsou převážně transmembránové proteiny, zatímco forma p68 je převážně proteinem cytoplazmatickým.

Ukázalo se tedy, že zatímco hlavními funkčními formami DR6 jsou proteiny p110 a p85, protein p68 pravděpodobně vůbec není žádnou kratší ani jinak modifikovanou formou receptoru DR6. Identitu proteinu p68 se nicméně nepodařilo prokázat...

V další části práce jsme se věnovali důvodům rozdílu molekulové hmotnosti receptoru DR6 zjištěné z jeho elektroforetické pohyblivosti a vypočtené z jeho aminokyselinové sekvence. Nadprodukce delečních mutantů DR6 ukázala, že modifikace zodpovědné za tento rozdíl jsou přítomné v extracelulární části DR6. Analýzou primární sekvence DR6 jsme zjistili, že obsahuje 6 potenciálních *N*-glykosylačních míst (motivů Asn-X-Ser/Thr) a s využitím empirických webových nástrojů pro analýzu aminokyselinových sekvencí jsme objevili též 6 pravděpodobných *O*-glykosylačních míst. Využitím dvou nezávislých přístupů pro studium proteinové glykosylace (inhibice enzymů glykosylačních drah a biochemická deglykosylace vhodnými glykosidázami) jsme zjistili, že hlavním důvodem zkoumaného rozdílu v molekulové hmotnosti DR6 skutečně je jeho masivní glykosylace. Dále se ukázalo, že zatímco forma p110 je *N*- i *O*-glykosylována, forma p85 je pouze *N*-glykosylovaný protein a je pravděpodobně pouze meziproduktem během syntézy plně modifikovaného receptoru DR6. Experimentálně jsme prokázali, že je modifikováno všech 6 potenciálních *N*-glykosylačních míst (tj. aminokyselinové zbytky Asn82, Asn141, Asn252, Asn257, Asn278 a Asn289), *O*-glykosylace se nám podařila zamapovat do oblasti Thr213 až Thr254, v níž leží všech 6 pravděpodobných *O*-glykosylačních míst (tj. aminokyselinové zbytky Thr213, Thr221, Thr227, Thr238, Thr245 a Thr254).

Jelikož dva proteiny skupiny receptorů smrti Fas/CD95 (Chakrabandhu et al., 2007) a DR4/TRAIL-R1 (Rossin et al., 2008), tedy receptory příbuzné DR6, již byly popsány jako palmitylované proteiny, zajímali jsme se též o tuto posttranslační modifikaci i v případě proteinu DR6. Sekvence receptoru DR6 obsahuje evolučně konzervovaný cysteinový zbytek (Cys368 v případě lidského DR6) v proximální části své cytoplazmatické domény, který je vhodným cílem pro *S*-palmitylaci. Experimenty založené na bodové mutagenezi tohoto aminokyselinového zbytku, autoradiografii imunoprecipitovaného proteinu DR6 s inkorporovaným radioaktivně značeným palmitátem a použití specifického palmitylačního inhibitoru prokázaly, že receptor DR6 skutečně je *S*-palmitylován na tomto cysteinovém zbytku.

Dále jsme se věnovali analýze funkcí výše popsaných posttranslačních modifikací receptoru DR6. Glykosylace aminokyselinových zbytků v extracelulárních částech transmembránových proteinů, včetně některých proteinů z rodiny receptorů TNFR, byly popsány jako modifikace ovlivňující jejich správné sbalení, lokalizaci a fyziologickou funkci (Mitra et al., 2006). V případě receptoru DR6 jsme ukázali, že *N*-glykosylace není vyžadována pro jeho lokalizaci na plazmatické membráně.

Všechna predikovaná *O*-glykosylační místa se nacházejí v oblasti mezi poslední doménou CRD a transmembránovou částí receptoru DR6. Tato oblast je bohatá na aminokyseliny prolin, serin a threonin a podobné sekvence se nacházejí i v některých dalších receptorech rodiny TNFR, jako je CD30, NGFR nebo TNFR2. Je známo, že delece této oblasti v receptoru NGFR mění směřování proteinu v polarizovaných buňkách na jejich apikální vs. basolaterální stranu (Monlauzeur et al., 1998; Yeaman et al., 1997). V případě receptoru DR6 delece této oblasti zcela narušila jeho transport na plazmatickou membránu, zřejmě na úrovni váčků Golgiho systému.

S-palmitylace receptorů Fas/CD95 (Chakrabandhu et al., 2007) a DR4/TRAIL-R1 (Rossin et al., 2008) byla ukázána jako významný faktor ovlivňující směřování těchto proteinů do membránových mikrodomén, tzv. lipidových raftů, což je nezbytný předpoklad pro jejich efektivní proapoptotickou signalizaci. Zjistili jsme, že v buňkách nádorových linií s vysokou expresí DR6 je tento receptor přítomen v lehkých membránových frakcích, které jsou nerozpustné v detergentu Brij98, ale rozpustné v detergentu NP40. Mutace palmitylačního místa v sekvenci DR6 ani použití specifického palmitylačního inhibitoru neměly na směřování receptoru do těchto mikrodomén žádný vliv. To by naopak mohlo být závislé např. na doméně DD jako v případě receptoru TNFR1 (Doan et al., 2004) nebo na *N*-glykosylaci jako např. v případě kanálu TRPM8 (Morenilla-Palao et al., 2009). Mutace *N*-glykosylačních míst v receptoru DR6 skutečně zabránily v jeho efektivním směřování do membránových mikrodomén, nicméně v případě použití *N*-glykosylačního inhibitoru jsme u endogenně exprimovaného DR6 tento efekt nepozorovali, takže vliv *N*-glykosylace receptoru DR6 na jeho směřování do membránových mikrodomén zůstává minimálně sporný.

IV.2. Aktivace T-lymfocytů spouští expresi receptoru DR6 závislou na transkripčních faktorech NF-κB a NF-AT

(komentář k publikaci Klíma M., Broučková A., Koc M., Anděra L.: T-cell activation triggers death receptor-6 expression in a NF-κB and NF-AT dependent manner. Mol Immunol, 2011)

Většina informací o funkci receptoru DR6 pochází ze zvířecího modelu, ze studia fenotypu DR6-deficientní myši. Na tomto modelu se ukázalo, že receptor DR6 hraje roli v regulaci imunitní odpovědi, a to zejména v inhibici proliferace a diferenciace CD4+ T-lymfocytů (Liu et al., 2002; Liu et al., 2001; Schmidt et al., 2003; Schmidt et al., 2005; Venkataraman et al., 2006; Zhao et al., 2001). Cílem této naší práce bylo srovnat myší a lidský model a popsat regulaci exprese DR6 na lidských primárních hematopoietických buňkách.

Pomocí metody průtokové cytofluorometrie jsme užitím našich vlastních monoklonálních protilátek analyzovali povrchovou expresi receptoru DR6 na lidských leukocytech z periferní krve a zjistili jsme, že žádná z testovaných subpopulací buněk neexprimuje DR6 v detekovatelném množství, a to včetně T- a B-lymfocytů, NK-buněk, monocytů, neutrofilních a eosinofilních granulocytů. Nicméně jsme zaznamenali významný nárůst exprese DR6 v T-lymfocytech stimulovaných různými faktory jako např. aktivační protilátkou proti CD3, PHA, nebo kombinací PMA a ionomycinu.

Publikovaná data ze studia DR6-deficientních myší naznačovala, že hlavní subpopulací myších T-lymfocytů exprimujících receptor DR6 by měly být CD4+ T-buňky. Naše experimenty na magneticky separovaných subpopulacích lidských T-lymfocytů však ukázaly, že po aktivaci exprimují DR6 jak CD4+, tak i CD8+ T-buňky, a to na úrovni mRNA detekované kvantitativní RT-PCR i na úrovni proteinové, detekované průtokovou cytofluorometrií.

Dále jsme se zajímali o mechanismus, který expresi receptoru DR6 po T-buněčné aktivaci reguluje. Stimulace T-lymfocytů obecně vede ke spuštění mnoha signálních drah, mezi nimiž významnou roli hrají signální dráhy vedoucí k aktivaci transkripčních faktorů z rodin NF-κB a NF-AT. Signální dráhy regulující aktivaci exprese DR6 jsme studovali za využití rozličných specifických inhibitorů a zjistili jsme, že skutečně jak inhibitory NF-κB dráhy (Bay 11-7085 a quinazoline), tak i inhibitory NF-AT dráhy (FK506 a cyklosporin A) významně snížily expresi DR6 po T-buněčné aktivaci. Podobný účinek měly i inhibitory signální dráhy PI3K-PKB/Akt (LY294002 a wortmannin) a v souladu s předchozím očekáváním též inhibitor kináz rodiny Src (PP2), inhibitor transkripce (aktinomycin D) a translace (cykloheximid). Naopak minimální, statisticky nevýznamný vliv na indukci exprese DR6 měly inhibitory kináz MAP-kinázových drah JNK1/2 (SP600125) a p38 (SB202190).

Experimentálně běžně používanou buněčnou linií pro studium T-receptorové signalizace jsou buňky T-buněčné leukemie Jurkat, které jsme v naší předchozí práci popsali jako DR6-pozitivní. Zajímalo nás tedy, jakým způsobem je regulována exprese receptoru DR6 v těchto buňkách po jejich stimulaci. Zjistili jsme, že aktivace buněk Jurkat vede k rychlému poklesu exprese DR6, na úrovni mRNA během 3-6 hodin, na úrovni proteinové během 2 dnů. Podobný účinek na expresi DR6 jako buněčná aktivace mělo u nestimulovaných buněk Jurkat použití inhibitorů signálních drah vedoucích k aktivaci transkripčních faktorů NF-κB a NF-AT a také inhibitorů signální dráhy PI3K-PKB/Akt. Jedním z hlavních důvodů rozdílné regulace exprese DR6 mezi primárními lidskými T-lymfocyty a buňkami linie Jurkat tak může být aberantní konstitutivní aktivace transkripčního faktoru NF-κB v buňkách Jurkat. Ta je pravděpodobně závislá na aktivitě protein-kináz PI3K a PKB/Akt (Kane et al., 1999), která souvisí s defektem v expresi fosfoinositid-fosfatázy PTEN v těchto buňkách (Shan et al., 2000).

V další části této práce jsme se věnovali analýze vazebných míst transkripčních faktorů NF-κB a NF-AT v promotoru DR6. K jejich vyhledávání jsme použili software Genomatix MatInspector (http://www.genomatix.de/products/MatInspector, (Cartharius et al., 2005)). Tato analýza *in silico* skutečně odhalila několik seskupení předpokládaných NF-κB- a NF-AT- vazebných míst v oblasti posledních 3 kb před počátkem transkripce DR6. Za použití luciferázových reportérů obsahujících různé deleční této oblasti DR6 promotoru jsme zjistili, že *cis*-regulační elementy exprese DR6 se nacházejí v oblasti promotoru DR6 obsahující proximální seskupení předpokládaných NF-κB- a NF-AT- vazebných míst. Bodové mutace těchto míst nicméně významný rozdíl v regulaci exprese DR6 nepřinesly, což mohlo být způsobeno buď vzájemnou redundancí těchto míst anebo pouze nepřímou rolí transkripčních faktorů NF-κB a NF-AT v regulaci exprese DR6.

Otevřenou otázkou pak zůstávají funkční důsledky exprese DR6 na aktivovaných T-lymfocytech. Přestože DR6 obsahuje doménu DD ("*death domain*") a bylo ukázáno, že se může účastnit aktivace buněčné smrti myších embryonálních nervových buněk (Nikolaev et al., 2009), nemusí být jeho fyziologická funkce na lidských primárních T-lymfocytech nutně spojena s apoptózou. Tomu nasvědčuje naše pozorování, že receptor DR6 nadprodukovaný v lidských buňkách je výrazně slabším iniciátorem buněčné smrti než jiné příbuzné receptory jako Fas/CD95, TNFR1 a TRAILové receptory TRAIL-R1/DR4 a TRAIL-R2/DR5, a dále již publikovaná skutečnost, že delece DR6 nijak neovlivňuje aktivací indukovanou buněčnou smrt (AICD) aktivovaných myších T-lymfocytů (Liu et al., 2001; Zhao et al., 2001). V tomto ohledu se receptor DR6 podobá jinému příbuznému proteinu, receptoru DR3, jehož hlavní funkcí je regulace diferenciace Th17 CD4+ T-lymfocytů (Jones et al., 2011).

Exprese receptoru DR6 na aktivovaných T-lymfocytech nemusí být sama o sobě postačující podmínkou pro spuštění DR6-dependentní signalizace, podobně jako u dalších příbuzných proteinů může být vyžadována aktivace příslušným ligandem. Jedinými dosud publikovanými ligandy receptoru DR6 jsou *N*-koncové odštěpené formy proteinů APP ("*β-amyloid precursor protein*") a APLP2 ("*amyloid precursor-like protein-2*"), které mohou během myší embryogeneze spouštět degeneraci axonů a buněčnou smrt míšních neuronů závislou na receptoru DR6 (Nikolaev et al., 2009). Protein APLP2 je exprimován na lidských primárních B-lymfocytech a monocytech, takže by jeho *N*-koncový fragment teoreticky mohl sloužit jako DR6 ligand, nicméně jeho interakci s DR6 *in vitro* ani *in vivo* s DR6 exprimovaným na lidských hematopoietických buňkách (aktivované primární T-lymfocyty, neaktivované buňky linie Jurkat) se nám nikdy nepodařilo detekovat žádnou z běžně užívaných metod, stejně jako DR6-dependentní aktivaci kaspázové kaskády nebo signálních drah vedoucích k aktivaci transkripčního faktoru NF-κB nebo stresových kináz z rodiny JNK. Důvodem pro toto pozorování může být rozdílnost mezi myším a lidským modelem anebo rozdílnost mezi nervovou a imunitní tkání, interakce mezi proteiny DR6 a *N*-APLP2 může např. vyžadovat přítomnost nějakého dosud neznámého koreceptoru přítomného pouze na embryonálních míšních neuronech apod.

V této naší práci se nám tedy podařilo přispět k charakterizaci regulace exprese receptoru DR6 na buňkách lidského imunitního systému, nicméně jeho funkce na těchto buňkách závislá na signalizaci spouštěné jeho dosud neznámým ligandem zůstává nadále neobjasněná.

IV.3. Adapterový protein ARAP1 se účastní mobilizace receptoru DR4 k plazmatické membráně

(komentář k publikaci Šímová Š., Klíma M., Čermák L., Šourková V., Anděra L.: Arf and Rho GAP adapter protein ARAP1 participates in the mobilization of TRAIL-R1/DR4 to the plasma membrane. Apoptosis, 2008)

Jedním z proteinů příbuzných receptoru DR6 je receptor DR4 ("*death receptor-4*", TNFRsf10a/TRAIL-R1/Apo2), který po vazbě svého ligandu TRAIL ("*TNF-related apoptosis-inducing ligand*", TNFsf10/Apo2L) může spouštět signalizaci vedoucí k aktivaci kaspázové kaskády a případně až k apoptóze. Regulace apoptózy indukované ligandem TRAIL skrze receptor DR4 probíhá na několika úrovních, přičemž jednou z nich je regulace množství a vzájemného poměru proapoptotických a antiapoptotických receptorů ligandu TRAIL na cytoplazmatické membráně cílových buněk. A právě tato úroveň regulace TRAILem indukované apoptózy se stala předmětem naší studie.

Naše práce začala vyhledáváním nových proteinů, které interagují s cytoplazmatickou částí receptoru DR4 a mohly by tak regulovat jeho signalizaci. Pro toto vyhledávání jsme použili metodu kvasinkového dvouhybridního systému a jako jednoho z kandidátů na nové interaktory s receptorem DR4 jsme objevili protein ARAP1 (centaurin-δ2), adaptérový protein vykazující aktivity Arf GAP a Rho GAP, tj. aktivity vedoucí ke stimulaci malých G-proteinů Arf a Rho, které se účastní vezikulárního transportu a organizace cytoskeletárních změn uvnitř buňky. ARAP1 má tři známé sestřihové varianty, přičemž námi objevená varianta interagující s receptorem DR4 neobsahuje produkt exonu 30, kterým je jedna z C-terminálních domén PH ("*pleckstrin homology*"). Další sestřihové varianty proteinu ARAP1 s receptorem DR4 neinteragovaly, stejně jako příbuzné proteiny ARAP2 a ARAP3.

V dalším kroku jsme interakci mezi proteiny DR4 a ARAP1 ověřili též v lidských buňkách. Oba proteiny, resp. jejich deleční mutanty, jsme naprodukovali v buňkách lidské nádorové linie HEK293T a pomocí jejich koimunoprecipitace jsme zjistili, že tyto proteiny po nadprodukci skutečně jsou přítomny ve stejném proteinovém komplexu a že pro interakci je důležitá proximální, k membráně přilehlá, doména intracelulární části receptoru DR4 a *C*-terminální část proteinu ARAP1. Kromě toho se ukázalo, že s proteinem ARAP1 slabě interaguje také receptor DR5, což je další receptor ligandu TRAIL sekvenčně příbuzný receptoru DR4.

Další nezávislý důkaz interakce proteinů DR4 a ARAP1 přinesla jejich vzájemná kolokalizace ve stejných buněčných kompartmentech. Nadprodukovali jsme tyto proteiny v nádorové linii NCTC a sledovali jejich lokalizaci po vystavení buněk působení ligandu TRAIL. V nestimulovaných

buňkách je receptor DR4 přítomen především na plazmatické membráně, zatímco protein ARAP1 v endoplazmatickém retikulu a Golgiho systému. V buňkách stimulovaných ligandem TRAIL oba tyto proteiny mění svoji lokalizaci a kolokalizují v ranných endozómech detekovaných protilátkou proti proteinu Rab5.

V další části práce jsme se věnovali funkčním důsledkům interakce mezi proteiny DR4 a ARAP1. Zjistili jsme, že transientní nadprodukce proteinu ARAP1 nemá žádný vliv na buněčnou lokalizaci receptoru DR4 ani na citlivost buněk k apoptóze indukované ligandem TRAIL. Naopak, po snížení exprese proteinu ARAP1 pomocí různých siRNA došlo v některých lidských nádorových liniích ke snížení množství receptoru DR4 lokalizovaného na plazmatické membáně a detekovaného průtokovou cytofluorometrií, anižby došlo k celkovému úbytku DR4 na úrovni mRNA nebo úrovni proteinové.

Navazující experimenty ukázaly, že úbytek receptoru DR4 lokalizovaného na plazmatické membráně vyvolaný potlačením exprese proteinu ARAP1 pomocí siRNA měl za následek též pomalejší nástup apoptózy vyvolané vystavením buněk působení ligandu TRAIL a kvantifikované pomocí detekce štěpení cytokeratinu-18, jednoho ze substrátů kaspázy-3. Obdobně zpožděné a zeslabené pak byly v těchto buňkách i další procesy spojené s apoptotickou signalizací, jako např. štěpení proteinů kaspáza-8, Bid a PARP, nebo fosforylace kinázy JNK.

Tato naše práce tedy přinesla objev nového interakčního partnera receptoru DR4, kterým je adaptérový protein ARAP1. Ten se kromě svých aktivit Arf GAP a Rho GAP může přímo účastnit vezikulárního transportu dalších proteinů mezi váčky Golgiho systému a plazmatickou membránou (Miura et al., 2002). Podobná schopnost již byla dříve popsána u proteinů příbuzných proteinu ARAP1, např. proteiny ASAP1 a ARAP3 regulují vezikulární transport receptoru EGFR (Kowanetz et al., 2004; Nie et al., 2006), anebo proteiny AGAP1 a AGAP2 ovlivňují recyklaci transferinového receptoru (Nie et al., 2005). Potlačení exprese proteinu ARAP1 v našem modelu skutečně vede k narušení transportu receptoru DR4 (a částečně i DR5) na plazmatickou membránu. Tento defekt byl již dříve popsán jako důvod rezistence střevních nádorových buněk SW480 k apoptóze indukované ligandem TRAIL (Jin et al., 2004). A i v našem případě potlačení exprese proteinu ARAP1 vedlo ke snížení apoptotické signalizace po vystavení buněk působení tohoto ligandu.

Tato naše práce tedy přispívá k objasnění mechanizmů ovlivňujících citlivost či rezistenci nádorových buněk k programované buněčné smrti indukované ligandem TRAIL prostřednictvím regulace vezikulárního transportu jeho receptorů.

V. Závěr

Smyslem této práce bylo přispět k objasnění funkce proteinu DR6 na molekulární úrovni. Jednotlivé, předem vytyčené cíle, se nám podařilo naplnit pouze částečně. Podařilo se nám funkčně charakterizovat strukturu DR6 včetně jeho posttranslačních modifikací i objasnit mechanizmy regulace exprese DR6 na hematopoietických lidských buňkách. Konkrétně jsme z prezentovaných publikací získali zejména tyto dílčí závěry:

(1) Protein DR6 se přirozeně vyskytuje ve 2 formách o molekulových hmotnostech cca 85kDa a 110kDa. Dominantní forma 110kDa je extenzivně *N*- i *O*-glykosylovaná, *N*-glykosylační místa se nám podařila zamapovat do jednotlivých aminokyselin, *O*-glykosylační místa pak do poměrně malé oblasti, o níž jsme zjistili, že je nezbytná pro transport DR6 na plazmatickou membránu. Protein DR6 je dále modifikován i *S*-palmitylací, která zřejmě (narozdíl od *N*-glykosylace) nemá vliv na směřování DR6 do membránových mikrodomén.

(2) Protein DR6 je exprimován na aktivovaných CD4+ i CD8+ T-lymfocytech. Jeho exprese je závislá na transkripčních faktorech NF-κB a NF-AT. Též jsme analyzovali roli jednotlivých predikovaných vazebných míst těchto transkripčních faktorů v DR6 promotoru. Leukemické buňky Jurkat, narozdíl od primárních T-buněk, exprimují DR6 před stimulací a po ní jeho expresi potlačují. Exprese DR6 na neaktivovaných buňkách Jurkat je závislá na konstitutivně aktivní signalizaci z kinázy PI3K.

Dalším dílčím cílem našeho studia receptoru DR6 bylo objevování jeho nových interakčních partnerů, a případně objasnění funkce těchto proteinů v signalizaci z DR6. S využitím metody kvasinkového dvouhybridního systému jsme objevili několik zajímavých proteinů interagujících s intracelulární částí DR6 v kvasinkách *Saccharomyces cerevisiae*. Tomuto tématu se již věnovaly diplomové práce (Klíma, 2003) a (Zájedová, 2006). Nezbytným předpokladem dalšího studia funkcí těchto interakcí v lidských buňkách jsou nástroje umožňující spouštět signalizaci z DR6, které jsme bohužel až do ukončení této disertační práce neměli k dispozici (viz níže)

Téma interakčních partnerů DR6 a jejich funkce v signalizaci z DR6 tedy není součástí této disertační práce, nicméně třetí prezentovaná publikace se věnuje tématu podobnému, a to funkční charakterizaci interakce mezi receptorem DR4 a adaptérovým proteinem ARAP1, která byla objevena právě pomocí kvasinkového dvouhybridního systému. Zjistili jsme, že receptor DR4 a protein ARAP1 spolu interagují v kvasinkách *S.cerevisiae* i v lidských buňkách, a že v buňkách vystavených působení ligandu TRAIL spolu kolokalizují v ranných endozómech. Snížení exprese proteinu ARAP1 vede ke snížení množství proteinu DR4 na plazmatické membráně, což má za

následek snížení citlivosti buněk k působení ligandu TRAIL, ke zpomalení průběhu apoptózy i dalších procesů spojených s apoptotickou signalizací. Protein ARAP1 je tak zřejmě důležitým regulátorem transportu receptoru DR4 z váčků Golgiho systému na plazmatickou membránu.

Jak již bylo zmíněno, jedním z hlavních cílů našeho studia receptoru DR6 bylo identifikovat jeho ligand(y), případně připravit agonistické monoklonální protilátky, a využít je ke studiu buněčné signalizace z DR6. Připravili jsme rekombinantní extracelulární část receptoru DR6 a pomocí průtokové cytofluorometrie jsme objevili nádorové buněčné linie exprimující povrchový antigen specificky vázající DR6, tedy potenciální DR6 ligand. Tento antigen se nám bohužel pomocí hmotnostní spektrofotometrie nepodařilo identifikovat.

Dále jsme připravili řadu monoklonálních protilátek proti extracelulární části receptoru DR6, a to izotypu IgG i IgM, nicméně žádné z těchto protilátek nebyly schopné (ani po prokřížení sekundární protilátkou) spustit obvyklé signální dráhy závislé na receptorech z rodiny TNFR, jako jsou kaspázová kaskáda, aktivace transkripčního faktoru NF-κB nebo stresových kináz z rodiny JNK. Všechny tyto dráhy přitom mohou být spuštěny nadprodukcí DR6 v některých nádorových liniích.

V roce 2009 bylo publikováno, že odštěpená N-koncová část proteinu APP ("*β-amyloid precursor protein*", *N*-APP) nebo jeho blízkého příbuzného APLP2 ("*amyloid precursor-like protein-2*", *N*-APLP2) mohou v míšních nervových buňkách myších embryí spouštět aktivaci kaspáz-3 a -6 závislou na receptoru DR6, což má za následek degeneraci axonů a buněčnou smrt. Zjistili jsme ale, že na našem modelu, tj. lidských buňkách hematopoietického původu exprimujících DR6, se proteiny *N*-APP a *N*-APLP2 na DR6 neváží a následně ani nespouštějí obvyklé signální dráhy spojené s proteiny rodiny TNFR. Důvody již byly diskutovány na straně 40, mohou spočívat v rozdílnosti mezi myším a lidským modelem anebo rozdílnost mezi nervovou a imunitní tkání, interakce mezi proteiny DR6 a *N*-APP/*N*-APLP2 může např. vyžadovat přítomnost nějakého dosud neznámého koreceptoru přítomného pouze na myších embryonálních míšních neuronech apod.

Další publikovaná data poukazující na možný dosud neidentifikovaný ligand receptoru DR6 pocházejí z práce (Derosa et al., 2007). Vystavení lidských primárních monocytů působení exogenního rekombinantního DR6 ovlivňovalo jejich *in vitro* diferenciaci do dendritických buněk pomocí IL4/GM-CSF. V naší práci (Klíma et al., 2009) jsme však ukázali, že toto pozorování bylo nejspíše artefaktem založeným na nespecifickém efektu použitého rekombinantního DR6 produkovaného v hmyzích buňkách.

Ligand receptoru DR6 exprimovaný buňkami lidského imunitního systému tak zůstává stále neznámý (pokud vůbec existuje). V souvislosti s tím zůstává nadále ne plně charakterizovaná buněčná signalizace spojená s receptorem DR6 a funkce proteinů interagujících s DR6 v této signalizaci. Zrovna tak ne zcela objasněná zůstává i samotná funkce receptoru DR6 na buňkách lidského imunitního systému. Případný budoucí objev ligandu receptoru DR6 tak může přinést zásadní průlom v jeho molekulární a funkční charakterizaci, a případně i budoucí terapeutické využití farmakologických nástrojů založených na receptoru DR6 a jeho ligandu a jejich použití v humánní medicíně.

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VII. Přílohy

(prezentované publikace)

Klíma M., Zájedová J., Doubravská L., Anděra L.: Functional analysis of the posttranslational modifications of the death receptor 6. Biochim Biophys Acta, 1793(10):1579-87, 2009.

Death receptor 6 (DR6/TNFRSF21) is a death domain-containing receptor of the TNFR superfamily with an apparent regulatory function in hematopoietic and neuronal cells. In this study we document that DR6 is an extensively posttranslationally modified transmembrane protein and that *N*- and *O*-glycosylations of amino acids in its extracellular part are mainly responsible for its approximately 40 kDa mobility shift in SDS polyacrylamide gels. Site-directed mutagenesis confirmed that all six extracellular asparagines are *N*-glycosylated and that the Ser/Thr/Pro cluster in the "*stalk*" domain juxtaposed to the cysteine-rich domains (CRDs) is a major site for the likely mucine-type of *O*-glycosylation. Deletion of the entire linker region between CRDs and the transmembrane domain, spanning over 130 amino acids, severely compromises the plasma membrane localization of DR6 and leads to its intracellular retention. Biosynthetic labeling with radiolabeled palmitate and side-directed mutagenesis also revealed that the membrane-proximal Cys368 in the intracellular part of DR6 is, similarly as cysteines in Fas/CD95 or DR4 ICPs, *S*-palmitoylated. However, palmitoylation of Cys368 is apparently not required for DR6 targeting into Brij-98 insoluble lipid rafts. In contrast, we show that *N*-glycosylation of the extracellular part might participate in directing DR6 into these membrane microdomains.

Klíma M., Broučková A., Koc M., Anděra L.:T-cell activation triggers death receptor-6 expression in a NF-κB and NF-AT dependent manner. Mol Immunol. 2011 Apr 16.

Death receptor-6 (DR6) apparently participates in the regulation of T-cell activation and/or activity as its genetic disruption results in enhanced CD4+ T-cell expansion, the production of Th2 cytokines, and interestingly also the compromised migration of CD4+ T cells to sites of inflammation. However, the mechanism of regulation of DR6 expression in cells of the immune system is not fully understood. In this communication we show that DR6 is not expressed in resting T cells from human peripheral blood or murine lymph nodes but that its expression is significantly upregulated in CD3 crosslinkingor PMA/ionomycin-activated T lymphocytes. DR6 expression is transiently increased in both activated human CD4+ and CD8+ T cells and it is apparently dependent on the activation of NF-κB and NF-AT signaling pathways. In contrast to primary peripheral blood T cells, the widely used model lymphoblastic leukemia T-cell line Jurkat is DR6-positive and unexpectedly, TCR-mediated stimulation of Jurkat cells strongly downregulates DR6 expression via suppression of its transcription.

Šímová Š., **Klíma M.**, Čermák L., Šourková V., Anděra L.: Arf and Rho GAP adapter protein ARAP1 participates in the mobilization of TRAIL-R1/DR4 to the plasma membrane. Apoptosis, 13(3):423-36, 2008.

TRAIL, a ligand of the TNFα family, induces upon binding to its pro-death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 the apoptosis of cancer cells. Activated receptors incite the formation of the death-inducing signaling complex followed by the activation of the downstream apoptotic signaling. TRAIL-induced apoptosis is regulated at multiple levels, one of them being the presence and relative number of TRAIL pro- and anti-apoptotic receptors on the cytoplasmic membrane. In a yeast two-hybrid search for proteins that interact with the intracellular part (ICP) of DR4, we picked ARAP1, an adapter protein with ArfGAP and RhoGAP activities. In yeast, DR4(ICP) interacts with the alternatively spliced ARAP1 lacking 11 amino acids from the PH5 domain. Transfected ARAP1 co-precipitates with DR4 and co-localizes with it in the endoplasmic reticulum/Golgi, at the cytoplasmic membrane and in early endosomes of TRAIL-treated cells. ARAP1 knockdown significantly compromises the localization of DR4 at the cell surface of several tumor cell lines and slows down their TRAIL-induced death. ARAP1 overexpressed in HEL cells does not affect their TRAIL-induced apoptosis or the membrane localization of DR4, but it enhances the cell-surface presentation of phosphatidyl serine. Our data indicate that ARAP1 is likely involved in the regulation of the cell-specific trafficking of DR4 and might thus affect the efficacy of TRAIL-induced apoptosis.

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Functional analysis of the posttranslational modifications of the death receptor 6

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ABSTRACT

Death receptor 6 (DR6/TNFRSF21) is a death domain-containing receptor of the TNFR superfamily with an apparent regulatory function in hematopoietic and neuronal cells. In this study we document that DR6 is an extensively posttranslationally modified transmembrane protein and that *N*- and *O*-glycosylations of amino acids in its extracellular part are mainly responsible for its approximately 40 kDa mobility shift in SDS polyacrylamide gels. Site-directed mutagenesis confirmed that all six extracellular asparagines are *N*-glycosylated and that the Ser/Thr/Pro cluster in the "stalk" domain juxtaposed to the cysteine-rich domains (CRDs) is a major site for the likely mucine-type of *O*-glycosylation. Deletion of the entire linker region between CRDs and the transmembrane domain, spanning over 130 amino acids, severely compromises the plasma membrane localization of DR6 and leads to its intracellular retention. Biosynthetic labeling with radiolabeled palmitate and side-directed mutagenesis also revealed that the membrane-proximal Cys368 in the intracellular part of DR6 is, similarly as cysteines in Fas/CD95 or DR4 ICPs, *S*-palmitoylated. However, palmitoylation of Cys368 is apparently not required for DR6 targeting into Brij-98 insoluble lipid rafts. In contrast, we show that *N*-glycosylation of the extracellular part might participate in directing DR6 into these membrane microdomains.

1. Introduction

Death receptor-6 was till recently one of the few "orphan" receptors of the TNFR superfamily. TNFR receptors are recognized by the presence of 1–4 cysteine-rich domains in their extracellular parts. Together with their ligands, predominantly expressed by hematopoietic cells, they participate in regulating the proliferation, survival or apoptosis of various, not exclusively hematopoietic, cells [1]. DR6 was identified as a new member of the death receptor subfamily, which is distinguished by the presence of an α-helical structural motif called the death domain in the intracellular part [2]. Some of the death receptors, such as Fas/CD95 or the TRAIL receptors TRAIL-R1/DR4 and TRAIL-R2/DR5, are potent inducers of apoptosis, while others such as DR3 or EDAR participate in the regulation of T cell activation or play a role during development [3,4].

Cysteine-rich domains of DR6 as well as its death domain and the C-terminal α-helical CARD-like region are highly conserved in vertebrates from zebrafish to humans. Overexpression of DR6 in some cell lines leads to apoptosis and/or to the activation of NFκB and stress kinases of the JNK/SAPK family [2,5,6].

The unavailability of the DR6 ligand has restricted studies examining DR6 function mainly to analyses of DR6 knockout mice. DR6 is not, similarly as other death receptors, required for the development or survival of mouse embryos, but its genetic inactivation enhanced the proliferation of CD4+ T cells and the production of Th2 cytokines [7,8]. Similarly, DR6-deficient B lymphocytes were hyperproliferative following various stimuli and showed attenuated stimulation-induced cell death [9]. The enhanced activation of DR6-deficient T and B cells was also reflected in more severe graft-versus-host disease induced by allogenic bone marrow transplantation into irradiated recipient mice [10]. The development and progression of autoimmune or allergic responses, such as experimental autoimmune encephalomyelitis or allergic airway inflammation, is attenuated in DR6 knockout mice [11,12]. However, recently the cleaved off extracellular part of APP or APLP2 proteins was reported to function as a DR6 ligand and to trigger DR6-dependent death of neurons and/or pruning of their axons [13].

DR6 expression is also markedly increased in several human tumor-derived cell lines. Increased DR6 expression in PC3 or DU145 prostate carcinoma cells is accompanied by enhanced NFκB signaling and increased $BclX_L$ expression [14]. In another prostate carcinoma cell line, LnCAP, DR6 expression was induced in a TNFα/NFκB-dependent manner. Increased DR6 expression on tumor cells could have functional consequences as matrix metalloproteinase-14 is able to cleave DR6 from the cell surface, and the shed extracellular part of DR6 was suggested to attenuate the *in vitro* differentiation of monocytes into dendritic cells, which can contribute to tumor evasion from the immune system [15].

In this communication we document that DR6 is *S*-palmitoylated on its membrane-proximal cysteine and its extracellular part is extensively *N*- and *O*-glycosylated. We show that these DR6 modifications are

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related to each other and might affect the plasma membrane localization of DR6.

2. Materials and methods

2.1. Plasmids and antibodies

The coding region of human DR6 was amplified by RT-PCR from total HeLa RNA, prepared by TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. The PCR product was cloned into pBluescript SK vector (Stratagene) and sequenced. For bacterial expression, the DR6 intracellular fragment corresponding to amino acids 371–655 was cloned into the pET-15b expression vector (Novagen), generating a construct with an N-terminal histidine tag. For expression in eukaryotic cells, DR6 cDNA was subcloned into the pcDNA3 vector (Invitrogen).

Site-directed mutagenesis was used to replace the asparagine residues of human DR6 at positions 82, 141, 252, 257, 287 and 289 with glutamines, the cysteine residue at position 368 with valine, and for internal deletions of the DR6 linker region (amino acids 212–349). Mutagenesis was performed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) with the primers summarized in Suppl. Table 2. Multiple point mutants were prepared by consecutive site-directed mutagenesis.

Mouse monoclonal antibodies against the extracellular part of DR6 were prepared using the standard techniques from splenocytes of mice immunized with the chimeric fusion protein of the extracellular part of DR6 (amino acids 42–335) and the Fc portion of human immunoglobulin IgG1 (Alexis). To raise mouse monoclonal or rabbit polyclonal antibodies directed against the intracellular domain of DR6, a purified His-tagged fragment of human DR6 (amino acids 370–655) expressed in E. coli was used as an immunogen. For some experiments, rabbit polyclonal antibody directed against the N-terminal peptide of mature DR6 (amino acids 42–56) was used as a control (BD PharMingen). Rabbit polyclonal anti-CD59 antibody (MEM-43) was kindly provided by Prof. V. Horejsi.

2.2. Cell culture, transfections and down-regulation of DR6 expression

The human T cell lines Jurkat and HuT78, the B cell lines KM3 and IM-9, the myeloid cell lines THP-1 and HL60 and the prostate carcinoma cell line LnCAP were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum. The human embryonic kidney cell lines HEK293 and HEK293FT, human immortalized keratinocytes NCTC and the cervical-carcinoma cell line HeLa were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. NCTC cells were provided by Dr. L. LoMuzio; all other cell lines were obtained from either ATCC or from the cell line collection of the Institute of Molecular Genetics.

Transfections of HeLa or HEK293 cells were performed using LF20020 (Invitrogen) or Fugene HD (Roche), as described by the manufacturer. Two independent DR6 siRNA oligonucleotides (Ambion) or control luciferase siRNA (Dharmacon) were used for lipofectamine RNAiMAX-mediated down-regulation of DR6 expression in NCTC cells (Suppl. Table 1). TNFα used for the treatment of LnCAP cells was purchased from R&D Systems.

2.3. Immunoprecipitation

The cells were harvested, washed with phosphate-buffered saline and lysed in ice-cold lysis buffer (1% NP-40, 20 mM Tris Cl pH 7.5, 100 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM Na_3VO_4, 10% glycerol), supplemented with protease inhibitors (Complete® protease inhibitor cocktail, Roche). After solubilizing for 30 min on ice, the lysate was pre-cleared by centrifugation at 16,000×*g* for 30 min. The resulting supernatant was incubated either with anti-DR6 mAb covalently coupled to CNBr-activated Sepharose beads (GE Healthcare) or with rabbit anti-DR6 antibodies bound to protein A/G Sepharose (Pierce) for 1 h at 4 °C. After three washes with 10 volumes of the lysis buffer, the immunoprecipitates were directly eluted with Laemmli sample buffer and analyzed by Western blotting using either rabbit or mouse anti-DR6 antibodies.

2.4. Confocal microscopy and flow cytometry

HeLa cells transfected with DR6 mutants and grown on cover slips were permeabilized in a −20 °C methanol bath for 10 min and then for 5 s in cold acetone. After washing in PBS the slides were incubated with anti-DR6 mAb for 30 min, followed by 30 min incubation with 4 μg/ml Alexa488-conjugated goat anti-mouse IgG (Molecular Probes). The samples were mounted in moviol with DRAQ5® (Axxora) and viewed with a Laserscan confocal microscope (Leica TCS SP).

The cells for flow cytometry analysis were harvested and incubated on ice with a blocking solution (PBS + 0.2% gelatine and 0.1% sodium azide=PBS-GA) containing 20% heat-inactivated human serum for 10 min. After blocking, the samples were incubated on ice in PBS-GA with anti-DR6 mAb (5 μg/ml) for 30 min, followed by a 30-min incubation on ice in PBS-GA with 4 μg/ml phycoerythrin-conjugated goat anti-mouse IgG1 (SouthernBiotech). After final washing, the surface expression of DR6 on living cells (Hoechst33258 low) was analyzed by flow cytometry on a LSRII (BD Biosciences).

2.5. Cell treatments and endoglycosidase digestions

The cells were cultured overnight in the presence of the N-glycosylation inhibitor tunicamycin (final concentration 5 μg/ml, Sigma), or in the presence of 2 mM benzyl-2-acetamido-2-deoxy-α-D-galactose (benzyl-*O*-GalNAc, Sigma), an inhibitor of the formation of *N*-acetylgalactosamine-*O*-Ser/Thr-linked glycoconjugates, or alternatively in the presence of 200 μM 2-bromopalmitate (Sigma), a palmitate analogue and an inhibitor of *S*-palmitoylation.

To perform endoglycosidase digestions, the immunoprecipitated DR6 was denatured and eluted from the anti-DR6-Sepharose beads by incubation in 1× Glycoprotein Denaturing Buffer (New England Biolabs) at 95 °C for 10 min. *N*-linked glycans were cleaved off with PNGase F (*N*-glycosidase F, NEB) and *O*-linked oligosaccharides with endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase, NEB) and neuraminidase (NEB) by 20 h incubation at 37 °C in 1× G7 Reaction Buffer (NEB) supplemented with 1% NP40.

2.6. Biosynthetic labeling with [3H]palmitate

HEK293FT cells (2×10^7 cfu) were transfected with DR6 expression plasmids, starved at 37 °C in 50 ml of plain RPMI1640 medium for 1 h and then 0.5 mCi of radiolabeled palmitate ([9,10(n)-3H]palmitic acid, specific activity 30–60 Ci/mmol; PerkinElmer Life and Analytical Sciences) was added. After 3 h at 37 °C the cells were washed and solubilized in a 1% NP40 lysis buffer, and the postnuclear supernatant was used for immunoprecipitation followed by SDS-PAGE. The wet gel was treated with Amplify solution (GE Healthcare) according to the manufacturer's recommendations, dried, and subjected to fluorography.

2.7. Density gradient ultracentrifugation

HEK293 cells (2×10^7 cfu) transfected with appropriate DR6 (wt or mt) expression plasmids or NCTC cells (10^8 cfu) were harvested, washed with PBS and lysed in 0.5 ml of ice-cold lysis buffer (1% Brij98, 20 mM Tris Cl pH 8.2, 100 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM Na_3VO_4), supplemented with protease inhibitors (Complete® protease inhibitor cocktail, Roche), on ice for 30 min. 0.5 ml of the lysate was then mixed at a 1:1 ratio with ice-cold 80% (wt/vol) sucrose diluted in lysis buffer and transferred into 5-ml ultracentrifugation

tubes, overlaid with 3.5 ml of ice-cold 30% sucrose diluted in lysis buffer and finally with 0.5 ml of sucrose-free lysis buffer. After centrifugation at 50,000 rpm in a MLS-50 rotor (cca 268,000 ×*g*; Beckman Coulter) for 18 h at 4 °C, 0.7 ml fractions were collected from the top to the bottom of each tube and analyzed by Western blotting.

3. Results

3.1. DR6 is expressed in two forms of apparent molecular masses 90 kDa and 110 kDa

Although from mouse knockout data the death receptor-6 is thought to have a dampening function in regulating the immune response, it was reported to be also expressed in some human, mainly prostate-derived, tumor cell lines [14]. In our screening for additional cells that might express DR6, we reproducibly observed in the cell lysates of all analyzed cell lines, of both hematopoietic and non-hematopoietic origin, a 68–70 kDa band (termed p70) that would correspond to the predicted molecular mass of human DR6 (68 kDa or 72 kDa including the putative signal peptide). This band was stained with polyclonal antibodies that map both to the N-terminal peptide (amino acids 42–56, e.g. Abcam ab8417) and to the cytoplasmic part of DR6 (amino acids 371–655, e.g. Abcam ab47180 or our own rabbit polyclonal antibody, marked by asterisks in Fig. 1A). However, wild-type DR6 exogenously expressed in HEK293 cells predominantly yielded higher molecular weight bands of apparent molecular masses 85–90 kDa (termed p90) and 105–110 kDa (termed p110), but not the p70 band (not shown). In addition, using our own monoclonal antibodies raised against the extracellular part of DR6, we were able to immunoprecipitate from some cell lines (KM3, Jurkat, NCTC) the 110 kDa and, to a lesser extent, the 90 kDa forms of DR6 (marked by arrows in Fig. 1A) but almost none of the 70 kDa protein. In order to resolve this uncertainty regarding the expression and apparent molecular weight of DR6, we also determined the cell surface expression of DR6 in the tested cell lines by flow cytometry (Fig. 1B) and quantified the relative amount of *DR6* mRNA by real-time qRT-PCR (Suppl. Fig. 1); these results closely matched those from the immunoprecipitation of the DR6 110/90 kDa forms, suggesting that the p70 protein is not the major functional form of DR6 and its real identity is uncertain (at least in cells that we used in this study).

These findings are supported by two other independent lines of experimental evidence. First, siRNA-mediated knockdown of *DR6* mRNA in NCTC cells led to the disappearance of only the 110/90 kDa forms and not the 70 kDa band (Fig. 1C). Second, according to the published observation that TNFα induces the expression of DR6 through the activation of NF-κB [14], the p90 and p110 DR6 forms were strongly upregulated by the cultivation of LnCAP prostate carcinoma cells in the presence of TNFα, whereas the expression of the p70 protein again remained unaffected (Fig. 1D). Summing these data together, it appears that not the calculated 70 kDa form but the 110 kDa and, to a lesser extent, the 90 kDa bands are the major if not the only naturally expressed forms of DR6.

3.2. DR6 is a palmitoylated protein with an extensively glycosylated extracellular part

The significant difference between the predicted (70 kDa) and actual (110 kDa and 90 kDa) molecular masses of DR6 suggests the presence of possible posttranslational modification(s) that modulates its relative mobility in SDS-PAGE. These modifications most probably reside in the extracellular region of DR6 as the deletion mutant lacking most of the DR6 intracellular part modulated its relative mobility in SDS-PAGE in a similar way as did wild-type DR6 (not shown). The TNFR superfamily members are often subjected to glycosylation, and therefore we searched the extracellular sequence of DR6 for potential *N*- and mucin-type *O*-glycosylation sites using NetNGlyc 1.0 and NetOGlyc 3.1 predictions, respectively [16]. Human DR6 contains six potential *N*-linked oligosaccharide chain sites (Asn82, Asn141, Asn252, Asn257, Asn278 and Asn289) and multiple potential *O*-linked oligosaccharide chain sites with a greater preference for Thr213, Thr221, Thr227, Thr238, Thr245 and Thr254 (Suppl. Fig. 2A).

For assessing the relative contribution of *N*- and *O*-glycosylation, we combined cell treatment with known inhibitors of *N*- and *O*-glycosylation, tunicamycin and benzyl-2-acetamido-2-deoxy-α-D-galactose (benzyl-*O*-GalNAc) respectively, with the in vitro deglycosylation of the immunoprecipitated DR6. For *in vitro* enzymatic deglycosylation, we used either PNGase F (*N*-glycosidase F), which removes *N*-linked oligosaccharides by cleaving between the innermost GlcNAc and Asn residues, or a mixture of neuraminidase and endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase), which removes the terminal sialic acid residues or desialyated core 1 and core 3 *O*-linked disaccharides attached to Ser/Thr residues, respectively.

Cell treatment with glycosylation inhibitors as well as enzymatic deglycosylation, either alone or in various combinations, led to a distinct but not complete reduction of the apparent molecular mass of DR6 (Fig. 2A). PNGase F and tunicamycin interventions significantly enhanced the mobility of both p110 and p90, indicating that these forms of DR6 contain *N*-linked oligosaccharides (Fig. 2A, lanes 5 and 13). In contrast, the *O*-glycosylation inhibitor benzyl-*O*-GalNAc and *in vitro* *O*-linked deglycosylation of the immunoprecipitated DR6 partly enhanced the mobility of only the p110 form, which argues for the presence of *O*-linked oligosaccharides only in the p110, presumably a more mature form of DR6 (Fig. 2A, lanes 4 and 7). Subsequent usage of both of these de-*O*-glycosylation interventions shifted mobility of the p110 form even further implicating non-overlapping modes of the action of these reagents (Fig. 2A, lane 8). Both enzymatic *N*-linked deglycosylation and tunicamycin treatment shifted the mobility of the p90 band to approximately 70 kDa, suggesting that p90 might be an *N*-only-glycosylated intermediate form of DR6 (Fig. 2A, lanes 5 and 13). The combination of tunicamycin, benzyl-*O*-GalNAc and *O*-glycolytic enzymes enhanced DR6 gel migration most efficiently (Fig. 2A, lanes 18 and 20). Thus, it appears that the p110 form of DR6 is both *N*- and *O*-glycosylated and that these glycosylations largely account for the apparent approximate 40 kDa shift in its calculated mobility.

Glycosylation prediction analysis points to six potentially *N*-glycosylated asparagines in the DR6 extracellular part and a cluster of *O*-glycosylated serines and/or threonines between amino acids 212 and 254 of the DR6 precursor (Suppl. Fig. 2A). Individual and consecutive mutagenesis of all asparagines (starting from Asn82) to glutamines in the DR6 extracellular part revealed that each of them is *N*-glycosylated (gradual shift in the apparent molecular weight), and the migration of the ultimate DR6 mutant reflected the DR6 mobility in tunicamycin-treated cells (Fig. 2B). These data lead us to conclude that all six asparagines in the extracellular part of DR6 are *N*-glycosylated.

As Fas/CD95 and TRAIL-R1/DR4 were recently described as palmitoylated proteins targeted to lipid rafts [17,18], we asked whether this membrane domain targeting- and trafficking-related posttranslational modification can also occur in DR6. Human and mouse DR6 proteins contain a conserved cysteine residue in the membrane-proximal intracellular region (Cys368 in the human DR6 precursor), which could be potentially *S*-palmitoylated (Suppl. Fig. 2C). HEK293FT cells were transfected with DR6 and DR6 (C368V) mutants, cultured in medium containing radioactive [3H] palmitate, lysed and immunoprecipitated with DR6 antibody. Autoradiography of the SDS-PAGE-separated immunoprecipitates revealed that [3H]palmitic acid is incorporated into DR6 and slightly more efficiently also into DR6(6xNQ) mutant but not into the DR6(C368V) mutant, indicating that Cys368 is indeed being palmitoylated (Fig. 2C, lanes 1–4). Specificity of DR6 palmitoylation was further confirmed using a competitive palmitoylation inhibitor 2-bromopalmitate (Fig. 2C, lanes 6, 7).

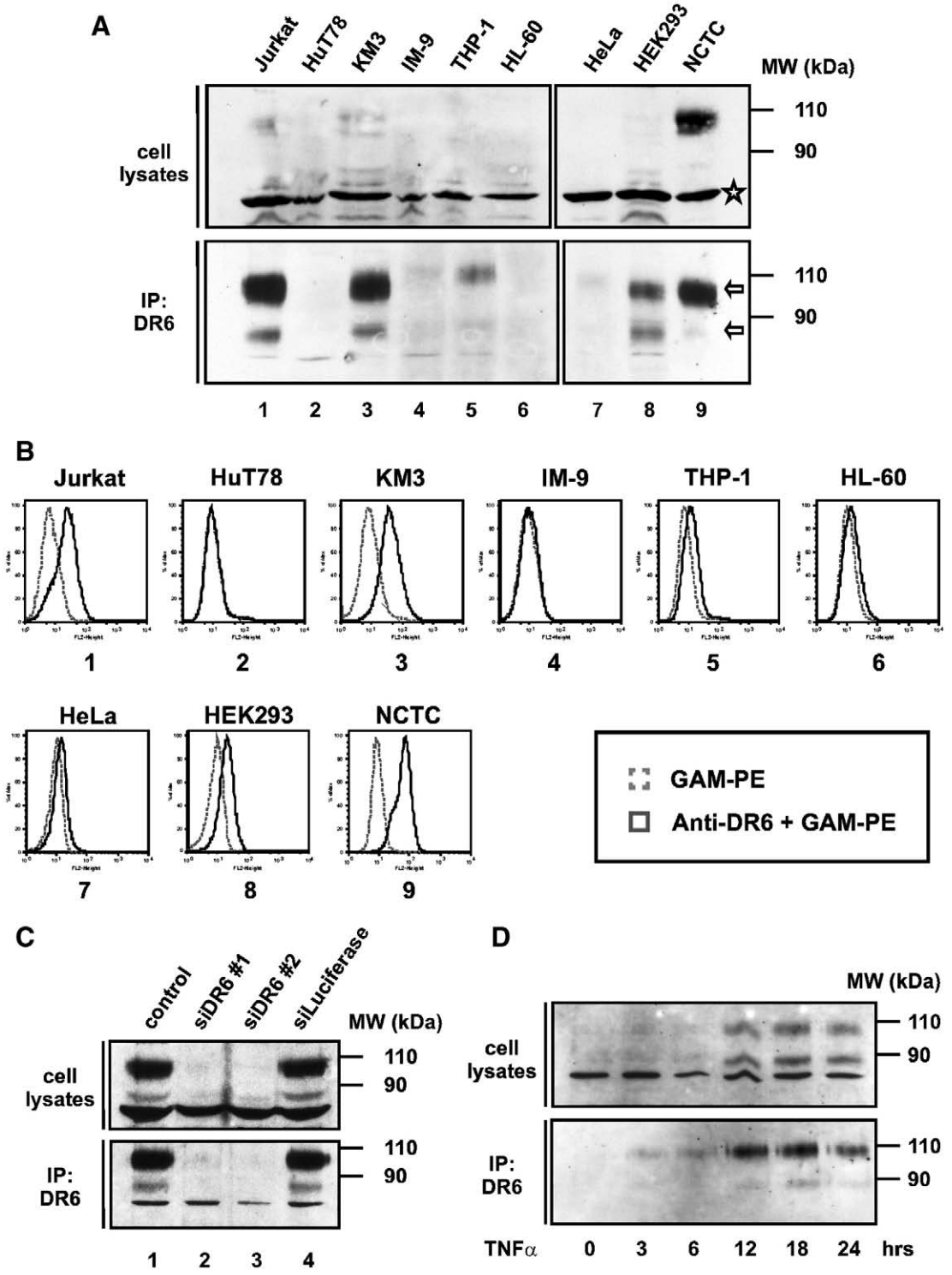


Fig. 1. Death receptor 6 is expressed at the cell surface of various cell lines as a 110 kDa protein. A. The indicated cell lines (approximately 3×10^7 cfu) were lysed and DR6 was either immunoprecipitated from the lysate with anti-DR6(ECP) mAb (lower panel) prior to Western blotting or the cell lysate was directly analyzed by Western blotting using rabbit anti-DR6(ICP) antibodies (upper panel). B. Cell surface expression of DR6 on these cell lines was analyzed by flow cytometry using anti-DR6(ECP) mAb. C. NCTC cells were transfected with control luciferase or two different DR6 siRNAs, and DR6 from their cell lysates was immunoprecipitated with anti-DR6(ECP) mAb and/or analyzed by anti-DR6(ICP) Western blotting. D. LnCAP prostate carcinoma cells were treated with TNFα (20 ng/ml) for the indicated time periods then lysed, and DR6 was immunoprecipitated with anti-DR6(ECP) mAb and revealed by Western blotting.

3.3. The extensively O-glycosylated linker region between DR6 CRDs and the transmembrane part is required for the plasma membrane localization of DR6

Having established the presence of multiple posttranslational modifications in DR6, we were eager to uncover their function. In addition to the already prepared mutants in the *N*-glycosylation and palmitoylation sites, we aimed to target the *O*-glycosylation sites as well. In contrast to most of its TNFR kin, DR6 harbors an unusually long (approximately 130 amino acids) linker region between CRDs and the transmembrane domain, which also contains the majority of the predicted mucin-type *O*-linked oligosaccharide chain sites, mainly

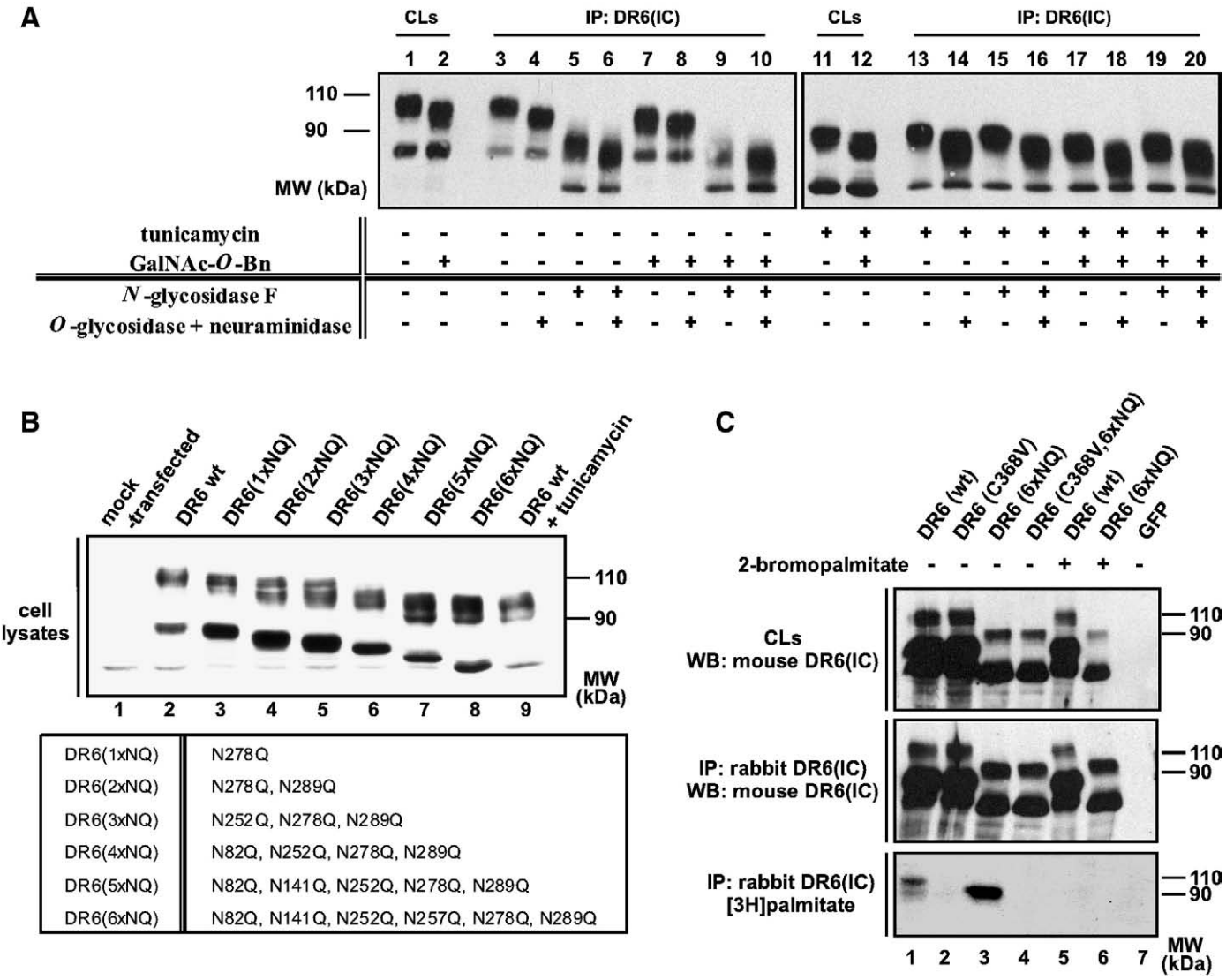
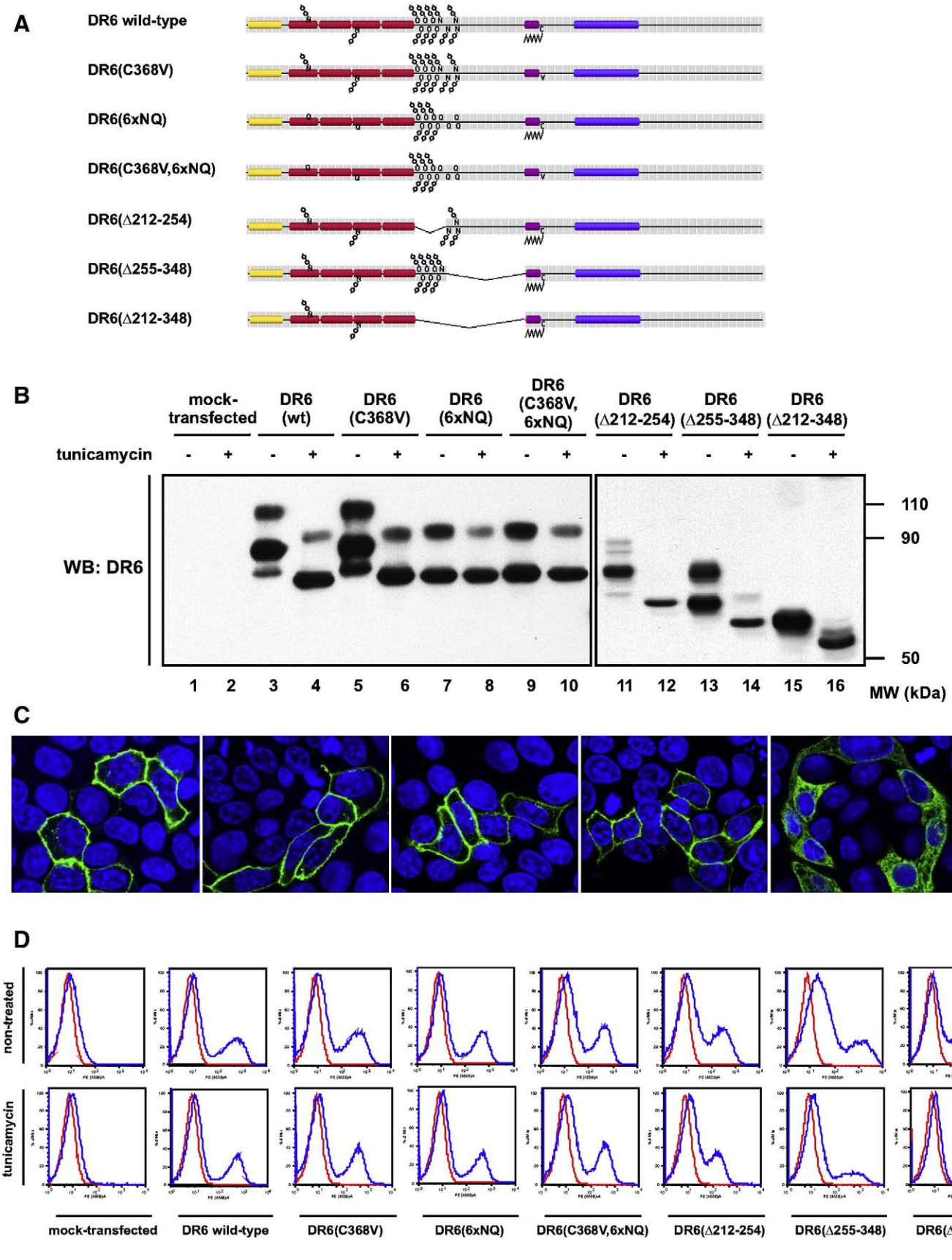


Fig. 2. DR6 is an extensively N- and O-glycosylated and S-palmitoylated protein. A. DR6-transfected HEK293FT cells were cultured with 5 μg/ml of tunicamycin and/or 2 mM benzyl-*O*-GalNAc for 15 h, lysed and then DR6 immunoprecipitated with mouse monoclonal anti-DR6(IC) antibody was, where indicated, subjected to glycolytic treatment and analyzed by Western blotting using rabbit anti-DR6(EC) polyclonal antibodies (Pharmingen). B. HEK293FT cells were transfected with plasmids encoding consequent DR6 (N→Q) mutants, lysed and analyzed by Western blotting with anti-DR6 rabbit pAbs. The mutated Asn in individual DR6 mutants are displayed in the inset. C. HEK293FT cells were transfected with the indicated DR6 expression plasmids and biosynthetically labeled with [3H]palmitate, then the DR6 in the cell lysates (upper panel) or the rabbit anti-DR6(IC) immunoprecipitates (middle and lower panels) were analyzed by SDS-PAGE followed by Western blotting with anti-DR6 antibodies and fluorography.

clustered between amino acids Thr212 and Thr254 (see Suppl. Fig. 2B). Similarly clustered Ser-/Thr-/Pro-rich sequences, called "stalk regions", were found in some other TNFR receptors (as TNFR2, CD30 or NGFR) and are also predicted to be extensively *O*-glycosylated (Suppl. Table 1). As multiple *O*-glycosylation sites are predicted with a higher probability mainly in the stalk region, we targeted it as well as its complementary part and the entire linker region for deletion. The complete set of DR6 mutants (Fig. 3A) was used for analyzing their effect on DR6 plasma membrane localization. The expression and proper folding of the DR6 variants transfected into HEK293FT cells were confirmed by immunoprecipitation with conformation-specific monoclonal antibodies to DR6 and by Western blotting. All proteins were expressed and, when applicable as revealed by tunicamycin treatment, also *N*-glycosylated. Mobility in SDS-PAGE and the membrane localization of the *S*-palmitoylation-deficient mutant DR6 (C368V) were not different from wild-type DR6, and the fully *N*-glycosylation-deficient mutant DR6(6xNQ) was also localized at the plasma membrane (Fig. 3). Deletion of the stalk region (amino acids 212–254) led to a large reduction in the amount of fully modified DR6 corresponding to the p110 form in wild-type DR6 and to the predicted shift in the mobility of the *N*-only-glycosylated form corresponding to the p90 form in wild-type DR6 (Fig. 3B, lanes 11–12). In contrast, removing the complementing sequence in the linker domain (amino acids 255–348) did not affect additional posttranslational modifications of DR6, arguing for the stalk domain as the major likely *O*-glycosylated region in the extracellular part of DR6 (Fig. 3B, lane 11). Notably, the efficient DR6/Δ255–348 presentation at the plasma membrane (Fig. 3D, compare the histograms of wild-type DR6 and DR6/Δ255–348) can be related to its enhanced *O*-glycosylation. As predicted, DR6/Δ212–348 contains just two *N*-glycosylation sites, and upon tunicamycin treatment it migrates in agreement with the calculated molecular weight of approximately 55 kDa (Fig. 3B, lanes 15–16). However, in contrast to the partial deletion mutants, removing the entire linker region from the DR6 extracellular part significantly affected the behavior of this DR6 mutant. It was drastically less mobilized to the plasma membrane than wild-type DR6 or the partial deletion linker region mutants and remained stacked most probably in the Golgi system (Fig. 3C, D). Thus, the linker region (or its major part) is required for the proper transport of DR6 to the plasma membrane.


A
DR6 wild-type
DR6(C368V)
DR6(6xNQ)
DR6(C368V,6xNQ)
DR6(Δ212-254)
DR6(Δ255-348)
DR6(Δ212-348)
B
mock-transfected
DR6 (wt)
DR6 (C368V)
DR6 (6xNQ)
DR6 (C368V, 6xNQ)
DR6 (Δ212-254)
DR6 (Δ255-348)
DR6 (Δ212-348)
tunicamycin
WB: DR6
110
90
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
MW (kDa)
C
D
non-treated
tunicamycin
mock-transfected
DR6 wild-type
DR6(C368V)
DR6(6xNQ)
DR6(C368V,6xNQ)
DR6(Δ212-254)
DR6(Δ255-348)
DR6(Δ212-348)

3.4. N-glycosylation but not S-palmitoylation of DR6 might affect its targeting to lipid rafts

The palmitoylation of Cys368 juxtaposed to the transmembrane region and the targeting of palmitoylated Fas/CD95 and TRAIL-R1 to lipid rafts raised a question about the plasma membrane sublocalization of DR6. To analyze in which membrane compartment endogenous DR6 resides, human keratinocyte-derived cells NCTC (the cell line with the highest DR6 expression that we detected) were lysed in Brij98 detergent and subjected to sucrose gradient ultracentrifugation. The p110 DR6 form was mainly detected in the same fractions close to the top of the gradient as was CD59, a well established marker of detergent-resistant glycosphingolipid-enriched microdomains (lipid rafts) (Fig. 4A). In contrast, the p70 protein was almost exclusively present in the dense non-raft fractions at the bottom of the gradient. To evaluate the role of DR6 *S*-linked palmitoylation, wild-type DR6 and a *S*-palmitoylation-deficient DR6(C368V) mutant were ectopically expressed in HEK293 cells and the cell lysates were subjected to sucrose gradient ultracentrifugation. In contrast to endogenous DR6, only part of the transfected wild-type DR6 was localized to the light lipid raft fractions (Fig. 4B, upper panel). The distribution of the DR6(C368V) mutant in the sucrose gradient fractions did not notably differ from that of wild-type DR6, arguing for a non-essential role of Cys368 palmitoylation in the lipid raft targeting of DR6 (Fig.4B, middle panel). In addition, the treatment of NCTC cells with 2-bromopalmitate, an inhibitor of *S*-linked-palmitoylation, did not affect its localization to the light lipid raft fraction (Fig. 4C). Thus, *S*-palmitoylation of DR6 is apparently not essential for DR6 targeting to the lipid rafts.

Several recent publications point to *N*-glycosylation as a possible targeting mark of transmembrane receptors to lipid rafts [19]. In support of these findings, we also observed *N*-glycosylation-dependent targeting of transfected DR6 into lipid rafts and discovered that the localization of the *N*-glycosylation-deficient DR6(6xNQ) mutant in the light, lipid raft-containing fractions was severely reduced (Fig. 4B, lower panel). However, the treatment of NCTC cells with tunicamycin, an inhibitor of *N*-glycosylation, did not influence the localization of DR6 to the light lipid raft fractions, arguing against a potential role of *N*-glycosylation in the mobilization of DR6 into lipid rafts (Fig. 4C, lower panel). Thus, the possible role of *N*-glycosylation in the targeting of DR6 into lipid rafts remains unresolved and should be further investigated.

A recently published report proposed a potential role of DR6 in the differentiation of monocytes into immature dendritic cells [15]. The authors, using recombinant, in insect cell-produced DR6-Fc fusion protein, partially suppressed the IL-4+GM-CSF-induced expression of the differentiation marker CD1a. We used this assay for evaluating the potential role of DR6 *N*-glycosylation. Monocytes from two different donors were differentiated into immature dendritic cells, and this differentiation could be suppressed by recombinant DR6-Fc fusion protein produced in a baculoviral system (Suppl. Fig. 3A). However, using either our recombinant or other commercially available DR6-Fc fusion protein produced in HEK293FT cells, we could not reproduce inhibition of IL-4+GM-CSF-induced monocyte differentiation imposed by the DR6-Fc produced in insect cells (Suppl. Fig. 3A). Such unexpected differences between these fusion proteins prompted us to compare their properties, including their mobility in SDS-PAGE. As shown in Suppl. Figs. 3B, C the baculovirally-produced DR6-Fc, despite its 15 amino acid extension in the DR6 extracellular part, migrated mainly due to the absence of most of the *N*-linked glycosylation in SDS polyacrylamide gel faster than DR6-Fc expressed in human cells.

4. Discussion

Death receptor-6 belongs among the longest (number of amino acids) and also the least characterized receptors from the TNFR family. Apart from the common cysteine-rich domains and the membrane-proximal death domain, it also contains an unusually long linker region in its extracellular part and the intracellular C-terminal CARD-like domain [20]. In this communication we show that DR6 is also a heavily posttranslationally modified receptor and that extensive and complex *N*- and *O*-glycosylation and potentially other modifications of its extracellular part are mainly responsible for a 40 kDa mobility shift in DR6 polyacrylamide gel migration, exceptional among TNFR superfamily members. We found that both transfected and endogenous DR6 is mainly expressed in 2 forms—the major *N*- and likely *O*-glycosylated p110 protein and the minor *N*-only-glycosylated form p90, which most probably represents a DR6 precursor transiently formed mainly upon the ectopic overexpression of DR6. In contrast to the clear-cut assessment of *N*-glycosylated sites in the extracellular part of DR6, even combined enzymatic and inhibitor-based treatment targeting mucin-type *O*-glycosylation led only to an approximate 25 kDa drop in the apparent molecular weight (Fig. 2A, lane 8), pointing to either the incomplete inhibition and/or accessibility of *O*-linked oligosaccharide chains or the presence of other posttranslational modifications such as xylose-*O*-Ser-linked or *N*-acetylglucosamine-*O*-Ser-linked glycosylation.

Glycosylation of amino acids in the extracellular parts of plasma membrane proteins, including the receptors of the TNFR family, can affect the folding, trafficking, localization and function of these receptors [21]. Defects in the *N*-glycosylation of some plasma membrane receptors such as the prostaglandin E2 receptor, the α chain of FcεRI, the dopamine D5 receptor, the HLD receptor SR-BI or the TRPM8 cation channel were reported to affect their folding, trafficking to the cell surface or targeting to submembrane domains such as lipid rafts [19,22–25]. In contrast to these receptors, we found that *N*-glycosylation of DR6 is apparently not required for its plasma membrane targeting as both a *N*-glycosylation-deficient DR6 mutant and DR6 from tunicamycin-treated cells were still transported to and localized at the cell surface. Also, our *N*-glycosylation-deficient DR6-Fc fusion protein was, in contrast to the *N*-glycosylation mutant of TNFR1, transported to the cell surface of HEK293FT cells and secreted (not shown and [26]). However, *N*-glycosylation deficient DR6 still maintains a high degree of *N*-glycosylation-independent posttranslational modifications, such as *O*-glycosylation, that can perhaps compensate for the loss of hydrophilicity and ensure proper folding and transport to the cell surface. Moreover, these results clearly show that *N*-glycosylation per se is not required for these additional posttranslational modifications.

In contrast to *N*-glycosylation, which is present partially in the cysteine-rich domains of DR6, mucin-type *O*-glycosylation (and other possible posttranslational modifications in DR6 p110 protein) take place exclusively in the linking the region between CRDs and the transmembrane part. Similar linker regions containing *O*-glycosylation-rich "stalk" domains are present in some other TNFR superfamily members such as CD30, NGFR or TNFR2. *O*-linked glycosylation of serines and/or

Fig. 3. The extracellular linker region between cysteine-rich domains and the transmembrane domain of DR6 is essential for plasma membrane targeting of DR6. A. Graphical bar view of DR6 mutants; yellow—signal peptide, red—cysteine-rich domains, violet—transmembrane region, blue—death domain; DR6(C368V)—palmitoylation-deficient mutant, DR6 (6xNQ)—*N*-glycosylation-deficient mutant. B. Wild-type and mutants of DR6 were expressed in HEK293FT cells with or without tunicamycin treatment and analyzed by Western blotting. C. HeLa cells were transfected with the indicated DR6 expression plasmids, and the cellular localization of DR6 was analyzed by confocal microscopy using purified anti-DR6 (ICP) polyclonal antibodies. D. HEK293FT cells were transfected with DR6 expression plasmids, stained with anti-DR6(ECP) mAb and the DR6 cell surface expression was analyzed by flow cytometry. (red histogram represents cells stained only with the secondary antibody and the blue one cells stained with anti-DR6(ECP) mAb).

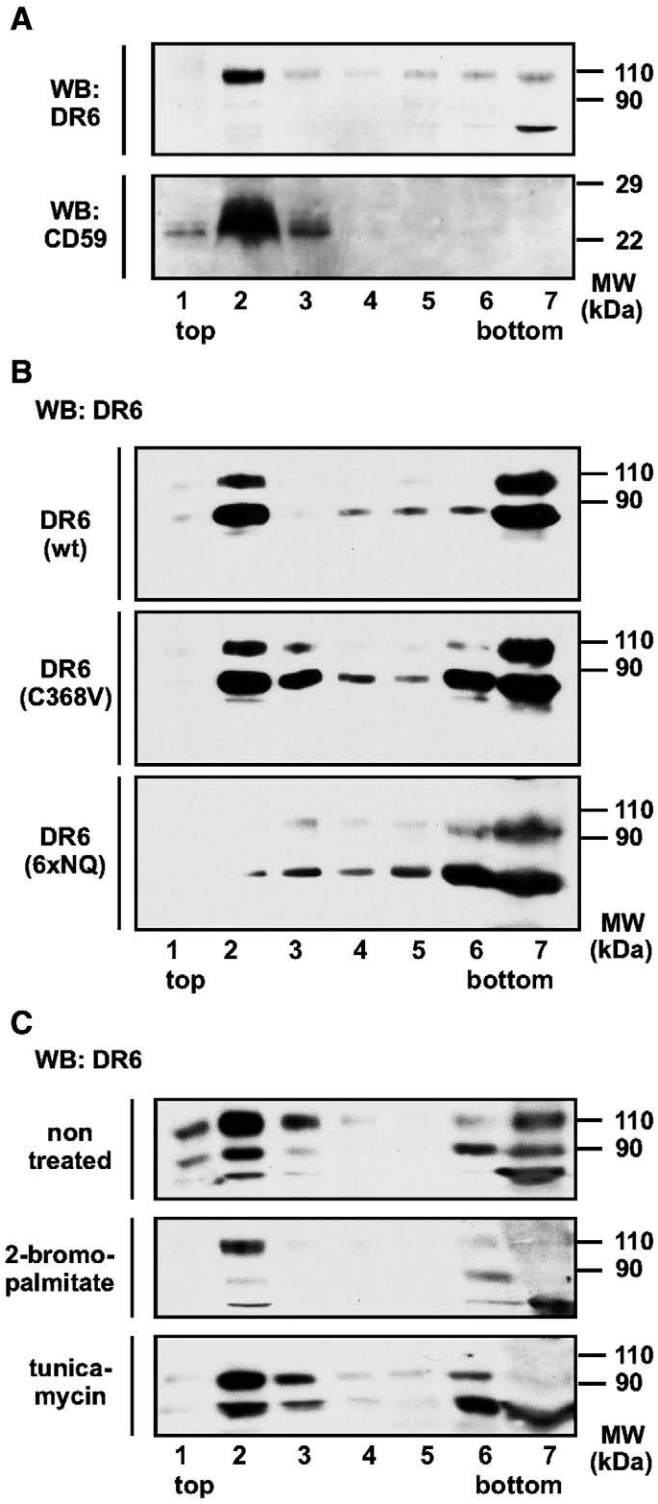


Fig. 4. *N*-glycosylation but not *S*-palmitoylation might participate in the targeting of DR6 into lipid rafts. A. NCTC cell lysate solubilized in Brij98 buffer was subjected to sucrose density gradient ultracentrifugation, and the fractions were analyzed by Western blotting using anti-DR6 or anti-CD59 (control marker of lipid rafts) antibodies. B. HEK293 cells were transfected with DR6 expression plasmids, solubilized in Brij98 buffer and analyzed as described in A. C. NCTC cells with or without 2-bromopalmitate or tunicamycin treatment were solubilized in Brij98 buffer and analyzed as described in A.

threonines in these domains could function as a pedestal for the proper orientation of CRDs towards the respective interacting ligand. In contrast to DR6, deletion of the linker region in NGFR did not affect the transport of NGFR to the plasma membrane but changed its apical-basolateral targeting in polarized cells [27,28]. Thus, the linker region might contain a sequential or structural motif required for the proper sorting of transmembrane proteins and/or, as we found for DR6, for their effective transport to the cell surface. DR6 lacking the entire linker region most probably remains stacked in the Golgi system.

In addition to plasma membrane targeting and localization, the glycosylation of DR6 can potentially regulate the interaction of DR6 with its recently described ligands APP or APLP2 [13]. Glycosylation was shown to both attenuate the interaction of FGFR1 with FGF-2 [29] and to enhance the ligand binding of the 5-HT3A receptor [30]. We also found apparent functional differences between insect and human cell-produced DR6-Fc proteins in respect to modulation of monocyte differentiation to dendritic cells. This discrepancy could be related to evident differences in *N*-linked glycosylation of the recombinant receptors produced in insect versus human cells (Suppl. Fig. 3C), hinting either at the functional significance of DR6 glycosylation status requirements for interaction with its ligand (possibly APLP2, which is expressed in human CD14+ monocytes, data from GNF Symatlas at http://symatlas.gnf.org/SymAtlas/) or at a non-specific off-target effect of the insect cell-produced, and possibly differently folded DR6-Fc. Thus, the role of posttranslational modifications in the interaction between DR6 and its ligand/-s remains to be explored.

An additional posttranslational modification present in Fas/CD95 and TRAIL-R1, but not in TNFR1 or TRAIL-R2, is *S*-palmitoylation of the membrane-proximal cysteine(s), which is apparently required for their association with lipid rafts and effective pro-apoptotic signaling [17,18]. We proved that similarly as these death receptors, DR6 is *S*-palmitoylated at the membrane-proximal Cys368 residue in the intracellular region. In cells expressing high levels of DR6 such as NCTC and PC3, the majority of DR6 is associated with the light, Brij98-insoluble (but NP40-soluble) lipid raft-containing membrane fraction. However, neither the treatment of cells with a palmitoylation inhibitor nor the usage of a DR6 point mutant lacking *S*-palmitoylation affected the subcellular localization of DR6, suggesting that other motifs might participate in DR6 targeting to Brij98-insoluble lipid rafts. Among possible lipid raft-targeting mechanisms could be the death domain-mediated targeting shown for TNFR1, the extracellular glycosphingolipid-binding motif found in Fas/CD95 or *N*-linked glycosylation-assisted targeting as shown for the TRPM8 cation channel [19,31,32]. Our *N*-glycosylation-deficient mutant of DR6 was inefficiently targeted to lipid raft fractions, suggesting a positive effect of *N*-glycosylation on DR6 localization to lipid rafts. However, as the inhibition of endogenous DR6 *N*-glycosylation in NCTC cells by tunicamycin did not significantly affect the distribution of DR6 in the lipid raft fractions, we cannot unambiguously attribute to the *N*-glycosylation of DR6 its lipid raft-targeting mark and surely other, yet unknown concurrent mechanisms can take over, when the N-linked glycosylation of DR6 is disabled in e.g. tunicamycin-stressed cells. Nevertheless, the lipid raft localization of DR6 could be important for the regulation and outcome of DR6 signaling. The lipid raft localization of TNFR1 was proven to be essential for the activation of NFκB, p42MAPK or RhoA signaling in a cell-specific manner [33–35].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:. 10.1016/j.bbamcr.2009.07.008.

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T-cell activation triggers death receptor-6 expression in a NF-κB and NF-AT dependent manner

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ABSTRACT

Death receptor-6 (DR6) apparently participates in the regulation of T-cell activation and/or activity as its genetic disruption results in enhanced CD4+ T-cell expansion, the production of Th2 cytokines, and interestingly also the compromised migration of CD4+ T cells to sites of inflammation. However, the mechanism of regulation of DR6 expression in cells of the immune system is not fully understood. In this communication we show that DR6 is not expressed in resting T cells from human peripheral blood or murine lymph nodes but that its expression is significantly upregulated in CD3 crosslinking- or PMA/ionomycin-activated T lymphocytes. DR6 expression is transiently increased in both activated human CD4+ and CD8+ T cells and it is apparently dependent on the activation of NF-κB and NF-AT signaling pathways. In contrast to primary peripheral blood T cells, the widely used model lymphoblastic leukemia T-cell line Jurkat is DR6-positive and unexpectedly, TCR-mediated stimulation of Jurkat cells strongly downregulates DR6 expression via suppression of its transcription.

1. Introduction

Death receptor-6 (reviewed in (Benschop et al., 2009)) is a death domain-containing receptor of the tumour necrosis factor-receptor family (Pan et al., 1998). TNFR receptors (reviewed in (Aggarwal, 2003)), together with their ligands, predominantly expressed by hematopoietic cells, participate in regulating the proliferation, survival or apoptosis of various, not exclusively hematopoietic, cells (Gaur and Aggarwal, 2003). Some of the death receptors, such as Fas/CD95 (Itoh et al., 1991; Oehm et al., 1992) or the TRAIL receptors TRAIL-R1/DR4 (Pan et al., 1997) and TRAIL-R2/DR5 (Walczak et al., 1997), are potent inducers of apoptosis, while others such as TNFR1 (Aggarwal et al., 1985) or DR3 (Chinnaiyan et al., 1996) participate in T-cell co-stimulation and in the regulation of T-cell-mediated inflammation (Meylan et al., 2008; Rothe et al., 1993). DR6 has been one of the few remaining orphan receptors of the TNFR family. Recently, a cleaved *N*-terminal fragment of the β-amyloid precursor protein (*N*-APP) was proposed as a DR6 ligand, triggering DR6-dependent axonal pruning and the cell death of spinal neurons during murine embryogenesis (Nikolaev et al., 2009).

Most of our current knowledge about the role of DR6 in cellular physiology comes from analyses of mice with inactivated expression of this death receptor. DR6 is not, similarly as other death receptors, required for proper embryogenesis, and DR6 knockout mice are viable, fertile and have no gross abnormalities of their major organs, including their brains (Liu et al., 2001; Zhao et al., 2001). The major differences between wild-type and DR6−/− mice have been observed in the immune system. DR6−/− CD4+ T cells have been found to hyper-proliferate in response to TCR-mediated stimulation or protein antigen challenge both *in vivo* and *in vitro* and to display a profound polarization toward a Th2 phenotype (Liu et al., 2001; Zhao et al., 2001). Similarly, DR6-deficient B lymphocytes hyper-proliferated in response to various stimuli and showed attenuated stimulation-induced cell death (Schmidt et al., 2003). The enhanced activation of DR6-deficient T and B cells was also reflected in a more rapid onset of graft-versus-host disease with increased severity induced by allogenic bone marrow transplantation into irradiated recipient mice (Liu et al., 2002). However, DR6 role in hematopoietic cells might not be impinged only to the regulation of B or T cell proliferation, as the DR6 knockout mice were also found to be highly resistant to both the onset and the progression of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis, most likely due to reduced VLA-4 expression and impaired migration (Schmidt et al., 2005). Similarly, DR6−/− mice were protected from the development of airway inflammation in the ovalbumin-induced mouse model of asthma (Venkataraman et al., 2006).

Abbreviations: DR, death receptor; PBT, peripheral blood T lymphocyte; TNFRsf, tumor necrosis factor-receptor superfamily.

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In this communication we report the results of studies elucidating the regulation of DR6 expression in hematopoietic cells. In contrast to previously published data about the regulation of DR6 expression in murine T and B lymphocytes (Liu et al., 2001; Schmidt et al., 2003; Zhao et al., 2001), we found that DR6 is not expressed in resting T and B cells from human peripheral blood or mouse CD4+ T cells from lymph nodes, but is significantly upregulated in activated T lymphocytes upon TCR-mediated stimulation in a NF-κB- and NF-AT-dependent manner. Furthermore, unlike primary lymphocytes, Jurkat T cells are DR6-positive and strongly downregulate DR6 expression upon TCR-mediated stimulation in a transcription-dependent manner.

2. Materials and methods

2.1. Cells and their cultivation conditions

Human peripheral blood mononuclear cells (PBMCs) were purified from buffy-coats from healthy donors by Ficoll-Paque density gradient centrifugation. Non-adhering T and NK cells were prepared from PBMCs after the removal of monocytes and B cells by adherence to tissue culture plastic and were subsequently referred to as crude peripheral blood T cells (PBTs). CD4+ and CD8+ T lymphocytes were prepared from PBMCs by magnetic separation with an autoMACS Separator (Miltenyi Biotech) according to the manufacturer's instructions. Magnetic depletion of other cell populations was performed with GAM MicroBeads (Miltenyi Biotech) and a cocktail of primary antibodies as follows: MEM15 (mIgG1 α hCD14), MEM168 (mIgM α hCD16), B-D3 (mIgG1 α hCD19), MEM188 (mIgG2a α hCD56), and MEM31 (mIgG2a α hCD8) for CD4+ T-cell isolation or MEM241 (mIgG1 α hCD4) for CD8+ T-cell isolation, respectively.

Murine CD4+ T cells were negatively selected from murine lymph nodes by magnetic separation with an autoMACS Separator using a mCD4+ T-Cell Isolation Kit II (Miltenyi Biotech) according to the manufacturer's instructions. Jurkat B10 T cells (a Jurkat clone derived from Jurkat/ATCC cells with an elevated stable expression of TCR) were obtained from the cell line collection of the Institute of Molecular Genetics. Both Jurkat and primary human and murine T cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin at 37 °C and 5% CO_2.

2.2. T-cell activation and inhibition of downstream signaling

For the *in vitro* activation of T cells, culture dishes were precoated with purified sterile anti-CD3 antibodies MEM-57 (human T cells) or 2C11 (mouse T cells) (10 μg/ml in phosphate buffered saline, PBS) for 1 hour at 37 °C. Dishes were washed twice with PBS, and T cells resuspended in RPMI1640 supplemented with 10% FBS were seeded at a concentration of 1×10^6/ml and cultured for various time periods. For PMA/iono stimulation, phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich; 1 ng/ml) and ionomycin (Sigma Aldrich; 1 μg/ml) were used.

The sources and the working concentrations of the chemical inhibitors were as follows: Bay 11-7085 (Sigma Aldrich; 20 μM), quinazoline (Merck/Calbiochem; 10 μM), FK506 (Cell Signaling; 10 μM), cyclosporin A (LC Laboratories; 10 μM), wortmannin (Sigma Aldrich; 4 μM), LY294002 (Sigma Aldrich; 50 μM), SP600125 (Merck/Calbiochem; 10 μM), SB202190 (Merck/Calbiochem; 10 μM), PP2 (Sigma Aldrich; 10 μM), actinomycin D (Merck/Calbiochem; 0,2 μg/ml).

2.3. Antibodies

Anti-DR6 antibodies were prepared as described in (Klima et al., 2009). The sources of the other antibodies were as follows: monoclonal antibodies to human CD3 (MEM-57), CD4 (MEM-241), CD8 (MEM-31), CD14 (MEM-15), CD16 (MEM-168), CD25 (MEM-181), CD56 (MEM-188) and mouse CD3 (2C11) were kindly provided by Prof. V. Hořejší (Prague, Czech Republic), CD19 (GenClone/Diaclone, clone B-D3), CD69 (ExBio, clone FN50), TNFR1 (R&D Systems, clone 16803), IκBα (Cell Signaling, L35A5), P-IκBα (Cell Signaling, 14D4), NFκB/p65 (Cell Signaling, C22B4), P-NFκB/p65 (Cell Signaling, 93H1), actin (Santa Cruz Biotechnology, clone C-11).

2.4. Real-time quantitative PCR

Total RNA from cultured cells was isolated using an RNeasy Plus Mini Kit (Qiagen), according to the manufacturer's instructions. Oligo dT-primed cDNA was prepared with M-MuLV Reverse Transcriptase (Fermentas). The reaction mixtures for quantitative PCR reaction contained the obtained cDNA, 1× LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) and 0,5 μM of each specific primer. The used intron-spanning oligonucleotides are summarized in Suppl. Table 1. Three "house-keeping" genes, *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *Succinate dehydrogenase complex*, *subunit A* (*SDHA*) and *β-actin* for human T cells and *β-actin, GAPDH* and *ubiquitin* (*UBB*) for murine T cells, were used as internal controls to standardize the quality of different cDNA preparations. The PCR reactions were performed in a LightCycler 480 Instrument (Roche Applied Science). The results were analyzed using the LightCycler 480 software package. The relative abundances of individual mRNAs were calculated from the average CT values of each triplicate after normalizing against the average level of the "house-keeping" genes and then adjusted against the minimal values in the series. Average values with standard deviations are shown.

2.5. Immunoprecipitation, Western blotting and flow cytometry

Immunoprecipitation, Western blotting and flow cytometry were performed as described in (Klima et al., 2009). In flow cytometry assays, the numbers in histograms represent the ratio of the median fluorescence intensity of an antibody-stained sample to the negative control.

2.6. Luciferase reporter gene assay

Fragments of the *DR6* gene promoter were amplified by PCR from chromosomal DNA, prepared from HEK293T cells using a ZR Genomic DNA II Kit (Zymo Research). The PCR product was cloned into pBluescriptSK vector (Stratagene), sequenced and recloned into the firefly luciferase reporter vector pGL3-Basic (Promega). The oligonucleotides used for cloning are shown in Suppl. Table 1. The AP-1, NF-κB and NF-AT *cis*-reporting plasmids were purchased from Stratagene.

Jurkat T cells were electroporated with these constructs and *Renilla* pRL-SV40 reporter vector (Promega) as an internal control using the Neon™ Transfection System (Invitrogen) according to the manufacturer's instructions with recommended electroporation parameters (pulse voltage 1.350 V, pulse width 10 ms, 3 pulses). Luciferase activities were determined 24 hours post-transfection (in some cases followed by 48 h stimulation with PMA/ionomycin) using the dual luciferase system (Promega), according to the manufacturer's instructions in a single tube Sirius luminometer (Berthold). All reporter gene assays were done in triplicates, and the activities are shown as average values with standard deviations

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from at least three independent experiments, normalized against the *Renilla* luciferase activities and mock-transfected cells.

3. Results

3.1. Activated human peripheral blood T lymphocytes upregulate DR6

DR6 is thought to have a dampening function in regulating the immune response (Liu et al., 2001, 2002; Schmidt et al., 2003, 2005; Venkataraman et al., 2006; Zhao et al., 2001), but a thorough analysis of the regulation of its expression in cells of the immune system has been missing. In our recent report we showed that DR6 is expressed in some tumour cell lines of both hematopoietic and non-hematopoietic origin, e.g. in Jurkat cells (Klima et al., 2009). Here, we embarked on a comprehensive analysis of DR6 expression in human primary hematopoietic cells. Flow cytometry analysis of DR6 expression on the cell surface of subpopulations of human peripheral blood leukocytes, including T lymphocytes, B lymphocytes, NK cells, monocytes, and neutrophil and eosinophil granulocytes, showed that DR6 is not expressed in any of these cells (not shown). The data from DR6-deficient mice suggest that DR6 function in the immune system could be more pronounced in activated T or B cells. Indeed, we found that the activation of human peripheral blood T cells by CD3 cross-linking or PMA/ionomycin co-treatment led to significant upregulation of DR6 at the cell surface (Fig. 1A).

Next, we examined the kinetics of DR6 expression in human T-cell population activated by CD3 cross-linking at both mRNA (quantitative RT-PCR) and protein levels (flow cytometry of the cell surface-expressed DR6). DR6 was, in contrast to the almost unchanged expression of TNFR1, upregulated in a time-dependent manner. The highest expression of DR6 was observed 24 h (mRNA, Fig. 1B) or 48 h (protein, Fig. 1C) post-treatment with anti-CD3 antibody, then both DR6 mRNA and protein levels gradually dropped. However, second CD3 cross-linking-mediated activation of these pre-activated T cells led to re-expression of DR6, but it has been also accompanied by pronounced cell death (nor shown). Interestingly, we noticed similar kinetics of upregulation of *DR6* mRNA also in negatively selected mouse CD4+ T cells (Suppl. Fig. 1).

The data from DR6-deficient mice suggested that CD4+ T cells could be the main subpopulation of T lymphocytes expressing DR6. To examine which subpopulations of human T cells can express DR6 in an activation-dependent manner, we used negative immunomagnetic separation and obtained pure fractions of CD4+ and CD8+ T cells. The quality of the preparations (purity >95%) was confirmed by both real-time qRT-PCR for CD4+ vs. CD8+ expression (Suppl. Fig. 2A) and by flow cytometry (Suppl. Fig. 2B). Surprisingly, we found that both human CD4+ and CD8+ peripheral blood T cells upregulate DR6 expression at the mRNA (Fig. 1D) and protein levels (Fig. 1E) upon their stimulation, either by CD3 cross-linking or PMA/ionomycin co-treatment. Moreover, CD8+ T cells exhibited higher CD3 crosslinking-dependent expression of DR6 than did CD4+ T cells. The cell surface expression of the CD69 lectin was used as an early lymphocyte activation marker.

3.2. DR6 expression on activated human T cells is regulated by nuclear factors NF-κB and NF-AT

The TCR-mediated stimulation triggers a complex set of signaling pathways, initially dependent on the activation of Src family kinases Lck and Fyn and consequently leading to the balanced activation of several transcription factors, among which nuclear factor-kappa B (NF-κB) and nuclear factor of activated T cells (NF-AT) play prominent roles in the downstream activation responses. To analyze which signaling pathways and transcription factors are involved in the regulation of the DR6 expression during T-lymphocyte activation, we examined an effect of a panel of various inhibitors including NFκB and NF-AT signaling pathway inhibitors Bay 11-7085 (an inhibitor of IκBα phosphorylation), quinazoline (an inhibitor of NF-κB transcriptional activation), FK506 and cyclosporin A (inhibitors of the calcineurin phosphatase) on activation-induced expression of DR6. Human peripheral blood T lymphocytes were pretreated with these inhibitors, then activated *in vitro* with anti-CD3 antibody, and the cell surface expression of DR6 in these cells was analyzed by flow cytometry. Interestingly, both NF-κB and NF-AT signaling pathway inhibitors significantly suppressed not only the T-cell activation-dependent upregulation of CD25 but also of DR6 (Fig. 2, upper panel). As expected, inhibition of the Src family kinases by PP2 inhibitor abolished CD3 crosslinking-mediated upregulation of both DR6 and the activation markers CD25/IL2R and CD69. Blocking PI3 kinase with wortmannin significantly suppressed CD25 expression and also negatively affected expression of DR6. Inhibition of JNK and p38 MAP kinase pathways did not affect the T-cell activation-dependent upregulation of DR6 or the activation markers at all (Fig. 2, lower panel). Blocking transcription with actinomycin D also significantly suppressed DR6 and CD25 expression, while post-translational increase of CD69 cell surface mobilization was unaffected.

3.3. NF-κB and NF-AT-mediated signaling is required for maintaining DR6 expression in Jurkat cells

The Jurkat T-cell leukemia cell line is widely used as a model cell line for TCR-dependent signaling, and we also recently described it as being DR6-positive (Klima et al., 2009). Thus, we asked whether DR6 expression in Jurkat cells is also regulated in an activation-dependent manner. We treated Jurkat cells *in vitro* either with anti-CD3 antibody or by PMA/ionomycin and examined the effect of these stimuli on DR6 expression at both mRNA and protein levels. The expression of the CD69 lectin was used as an early T-cell activation marker. Surprisingly, we found that in these stimulated Jurkat cells, DR6 expression was strongly and rapidly downregulated (Fig. 3A–C). A significant decrease in *DR6* mRNA levels occurred within 3–6 h (Fig. 3A), whereas DR6 protein expression was diminished within 2 days (Fig. 3B and C). The time-course of the changes in *DR6* mRNA levels upon CD3 cross-linking strongly correlated with that after treatment with actinomycin D, suggesting that Jurkat stimulation via TCR-related signaling leads to the relatively rapid suppression of DR6 expression at the transcriptional level (Fig. 3D).

Having established a role for NF-κB and NF-AT in the regulation of DR6 expression during primary T-cell activation, we were interested in determining whether these transcription factors also participate in the regulation of DR6 expression in Jurkat cells. Interestingly, we found that similarly as the stimulation of Jurkat cells via CD3 cross-linking or PMA/ionomycin treatment, blocking either NF-κB or NF-AT signaling by chemical inhibitors resulted within 1–2 days in nearly complete suppression of DR6 expression at the cell surface (Fig. 3E, upper panel). Furthermore, DR6 expression was also suppressed by inhibitors of PI3K-Akt signaling pathway wortmannin and LY294002 (and as expected also by transcription or translation inhibitors actinomycin D or cycloheximide, respectively), but not by chemical inhibitors of JNK and p38 MAP kinase pathways neither by inhibition of Src family kinases (Fig. 3E, lower panel).

The apparent discrepancy in the regulation of NF-κB-dependent DR6 expression between stimulated PBTs and Jurkat cells led us to further analyze the activation-dependent status of some

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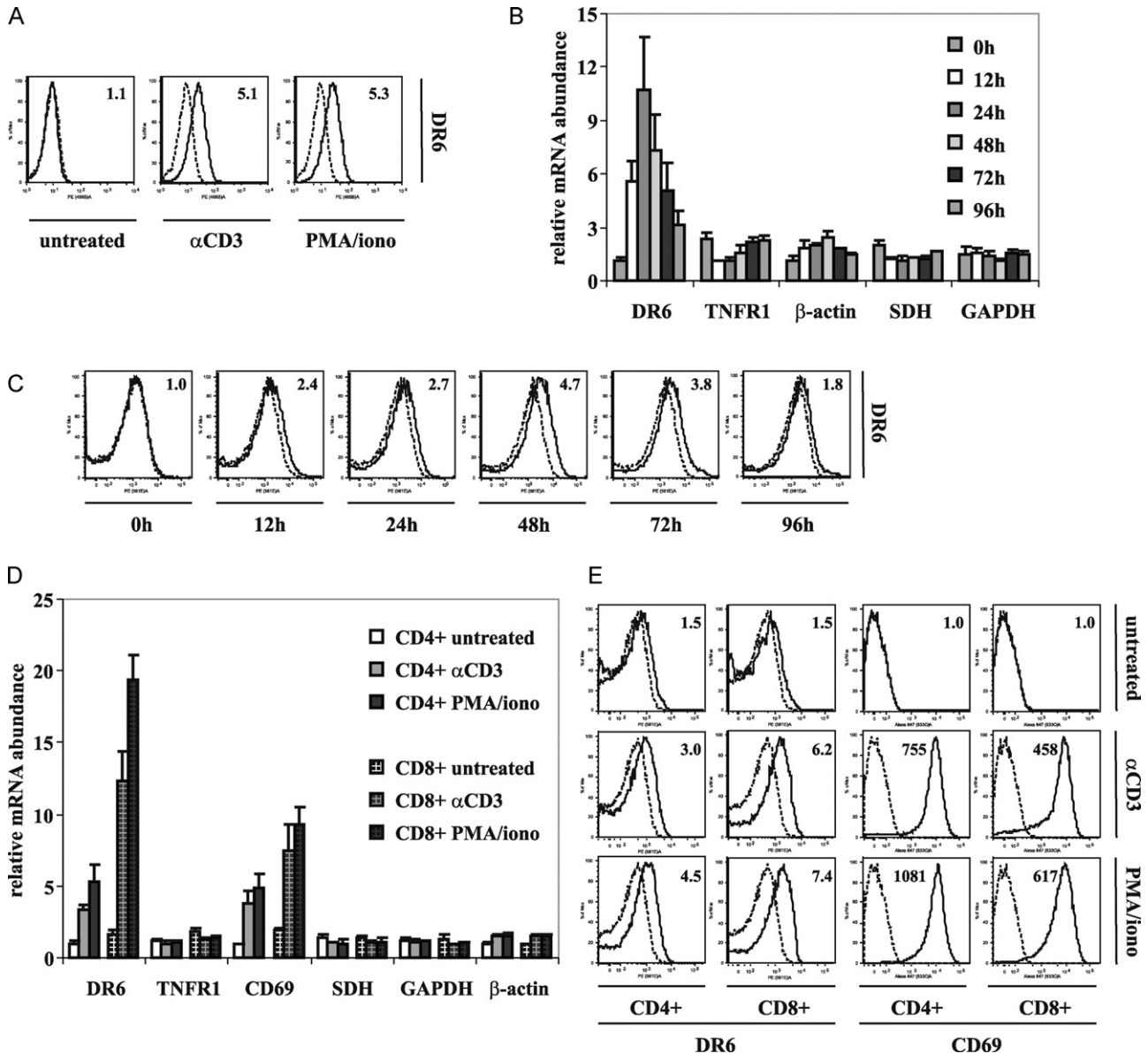

Fig. 1. Death receptor-6 is upregulated in activated human T lymphocytes (A) Human PBTs isolated by Ficoll-Paque gradient from buffy-coats were stimulated with either anti-CD3 antibody or PMA/ionomycin for 48 h. The cell surface expression of DR6 was analyzed by flow cytometry. The dashed lines in histograms represent cells stained only with the secondary antibody (negative control). The numbers in histograms state the ratio of the median fluorescence intensity of the DR6-stained (A,C) or CD69-stained (E) sample to the negative control. (B) Human PBTs were stimulated with anti-CD3 antibody for various time periods, and the relative expression of the indicated genes was determined by RT-qPCR as described in Materials and Methods. (C) The cell surface expression of DR6 on these cells was analyzed by flow cytometry using anti-DR6 monoclonal antibody 4G7. (D) Human CD4+ and CD8+ PBTs were isolated from buffy-coats by magnetic depletion of other cell populations using a cocktail of MEM antibodies (see Section 2) and stimulated with either anti-CD3 antibody or PMA/ionomycin for 48 h. The relative abundance of the indicated genes was determined by RT-qPCR. (E) The cell surface expression of DR6 (left panel) and CD69 (right panel) on CD4+ and CD8+ PBTs before and 48 h after stimulation was analyzed by flow cytometry.

critical components of the NF-κB signaling pathway in these cells. Stimulation of human PBTs led to the activation of NF-κB signaling, epitomized by the phoshorylation of both IκBα and also of the p65 subunit of the NF-κB complex (Fig. 3F, left panel), whereas in Jurkat cells the phosphorylation status of IκBα or NF-κB(p65) remained unchanged (Fig. 3F, right panel). Moreover, Jurkat cells transfected with AP-1, NF-AT or NF-κB luciferase reporters and stimulated with anti-CD3 antibody showed a significant activation of NF-AT dependent transcription but also a high basal NF-κB activity, which slightly dropped upon CD3 cross-linking (Fig. 3G). Taken together, these data suggest that the aberrant constitutive activation of NF-κB-, NF-AT- and PI3K/Akt- signaling pathways in non-stimulated Jurkat cells might be related to the maintenance of DR6 expression in these cells.

3.4. Other TNFR family members have different expression profiles in activated PBTs and Jurkat cells

The apparent discrepancy between peripheral blood T cells and Jurkat cells in regard to the regulation of DR6 expression prompted us to examine CD3 crosslinking-stimulated changes in the mRNA expression of other receptors from the TNFR family that were previously shown to participate in regulating the proliferation and activation of T cells. We could confirm data from a number of previous publications, documenting that CD3 crosslinking induced

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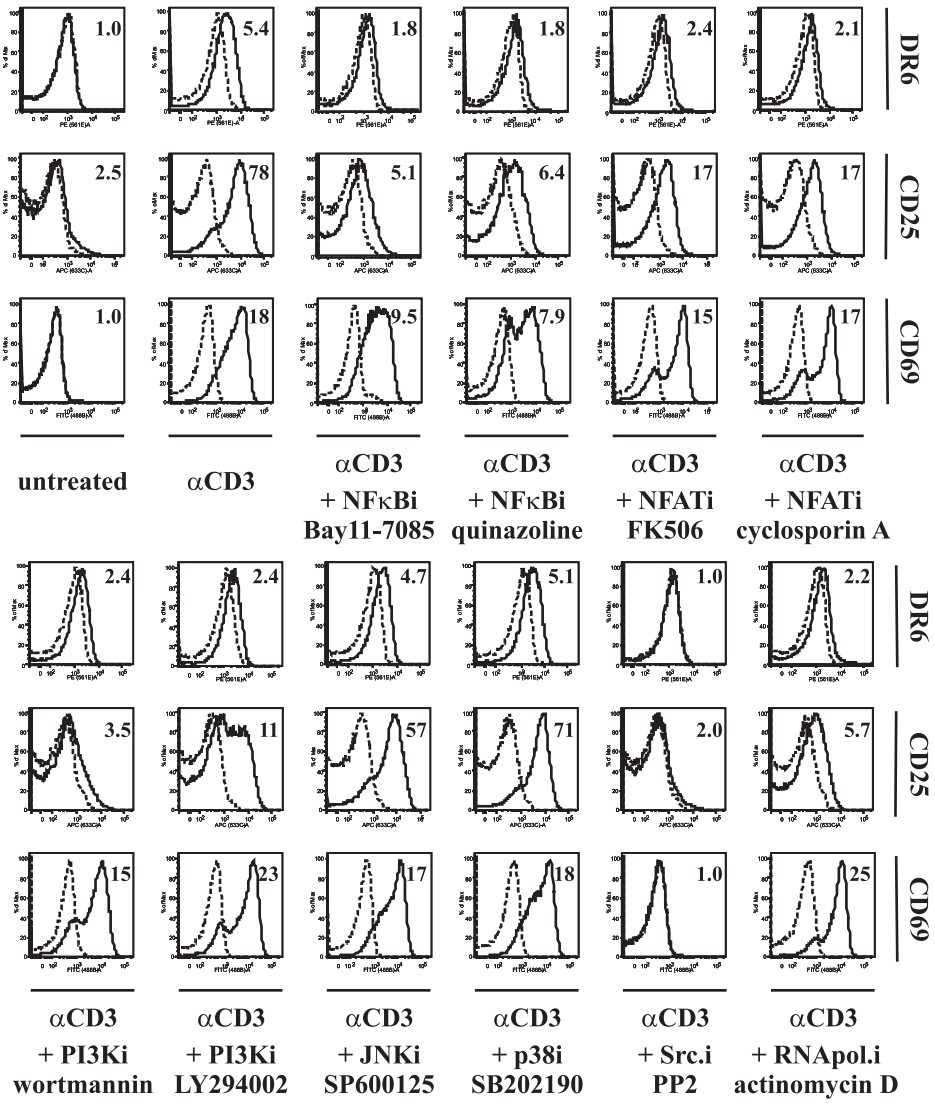

Fig. 2. Inhibition of NF-κB and NF-AT pathways attenuates the activation-dependent expression of DR6 in T cells Human PBTs were pretreated with the indicated inhibitors for 1 h, and then stimulated with anti-CD3 antibody for 24 h. The cell surface expression of DR6, CD25 and CD69 was analyzed by flow cytometry. As in Fig. 1, the numbers in histograms show the ratio of the median fluorescence intensity of the primary antibody-stained sample to the negative control.

the rapid and strong transactivation of CD30, OX40, GITR and 4-1BB receptors in human PBTs (Suppl. Fig. 3A). Also, in addition to the increased DR6 expression, GITR expression was upregulated in PBTs. Interestingly, the expression of both pro-apoptotic TRAIL receptors DR4 and DR5, and HVEM decreased in a time-dependent manner. In Jurkat cells GITR, OX40 and 4-1BB expression also increased upon CD3 stimulation in a time-dependent manner; however, DR6 expression, as we already showed, decreased (Suppl. Fig. 3B). This contrast in the CD3 crosslinking-stimulated expression of DR6 between PBTs and Jurkat cells is also underscored by the opposite expression profiles of HVEM, DR4 and CD27 (downregulation in PBTs and upregulation in Jurkat cells).

3.5. Analysis of the NF-κB and NF-AT binding sites in the DR6 promoter

We showed that both NF-κB and NF-AT signaling participate in the activation or maintenance of DR6 expression in T cells. As both of them are transcription factors, we used Genomatix MatInspector software (http://www.genomatix.de/products/ MatInspector (Cartharius et al., 2005)) to search for their binding sites in the DR6 promoter. Indeed, we found several clusters with putative NF-κB and NF-AT binding sites in the 3 kb region upstream from the DR6 transcription initiation site (TIS, Fig. 4A).

Then, we prepared a set of luciferase reporter vectors containing the DR6 TIS and either 3 kb of the upstream region (both proximal and distal NF-κB/NF-AT clusters) or its shorter version containing only the proximal NF-κB/NF-AT cluster (scheme in Fig. 4B, left panel). Jurkat cells were electroporated with these reporter vectors, stimulated either by CD3 cross-linking or with PMA/ionomycin treatment, and the activity of DR6 promoter-driven firefly luciferase was measured. Both the longer and shorter promoter variants showed similar basal activity, and both of them also exhibited a drop in luciferase activity upon stimulation, similar to the suppression of the expression of endogenous DR6 (Fig. 4B, lanes 2–3), suggesting that the *cis*-regulating elements of DR6 expression are present in the promoter proximal NF-κB/NF-AT cluster. Further analysis of point mutants of the DR6 promoter with mutated predicted NF-κB and NF-AT sites in this 1 kb promoter proximal region did not reveal any significant changes in the decrease of luciferase activities upon cell stimulation (Fig. 4B, lanes 4–6), suggesting a redundancy in NF-κB/NF-AT binding sites or an indirect role of these factors in the maintenance of DR6 expression.

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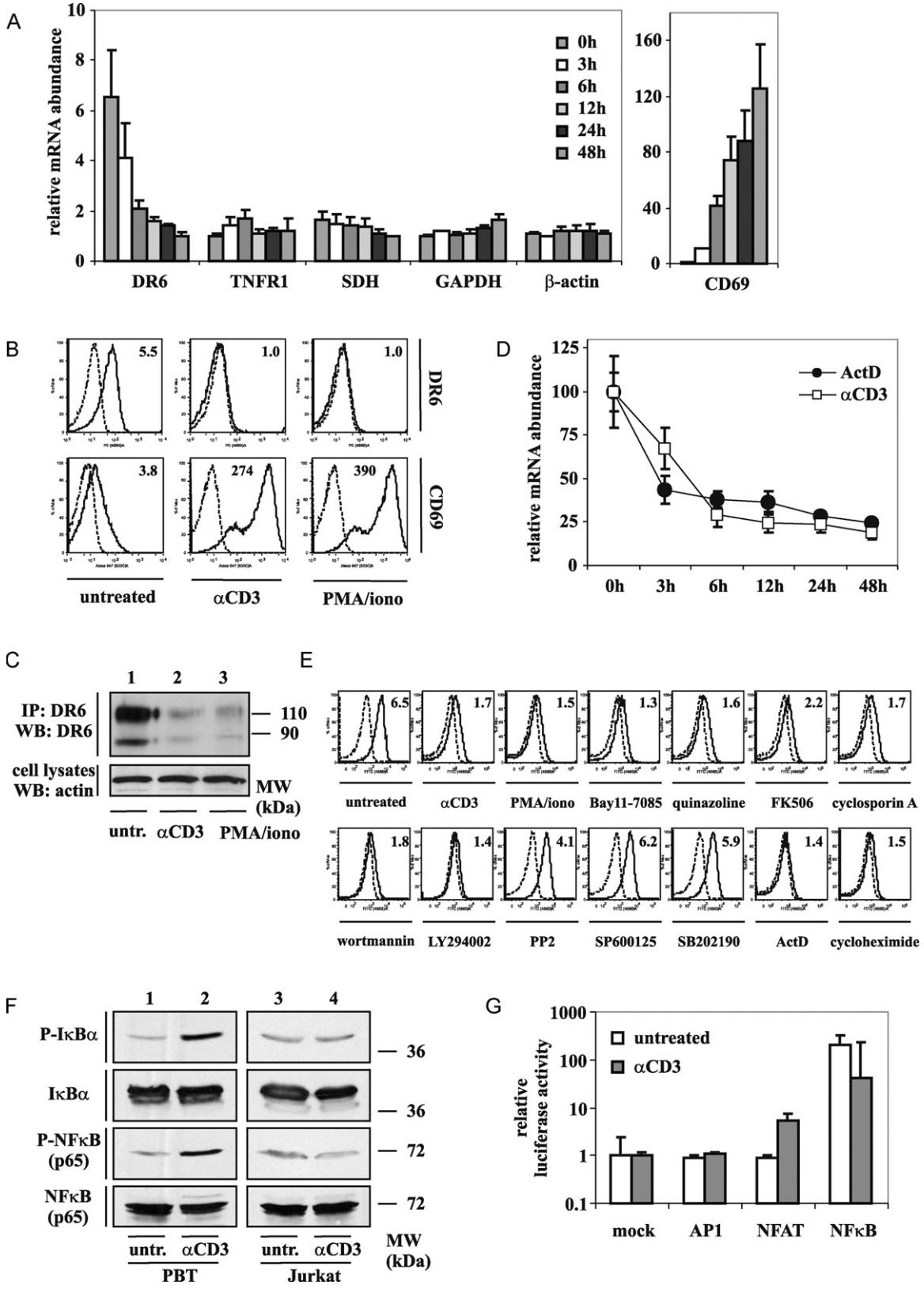

Fig. 3. DR6 is downregulated in Jurkat cells upon stimulation (A) Jurkat B10 T cells were stimulated with anti-CD3 antibody for 48 h and the relative expression of the indicated genes was determined by RT-qPCR. (B) The cell surface expression of DR6 (upper panel) and CD69 (lower panel) on Jurkat cells before and after stimulation for 48 h was analyzed by flow cytometry. (C) Jurkat cells (approximately 3×10^7 cfu) before and after stimulation for 48 h were lysed, and DR6 was either immunoprecipitated with anti-DR6 antibody 4G7 or the cell lysate was directly analyzed by Western blotting with the indicated antibodies. (D) Jurkat cells were either stimulated with anti-CD3 antibody or treated with actinomycin D for the indicated time periods. The relative abundance of *DR6* gene was determined by RT-qPCR. (E) Jurkat cells were incubated with the indicated inhibitors for 48 h, and the cell surface expression of DR6 was analyzed by flow cytometry. (F) Human PBTs (left panel) or Jurkat cells (right panel) before and after stimulation for 48 h with anti-CD3 antibody were lysed, and the cell lysates were analyzed by Western blotting with the specified antibodies. (G) Jurkat cells were electroporated with the indicated reporter plasmids, and the relative AP1/NF-κB/NF-AT-driven luciferase activities were determined after 48 h stimulation with anti-CD3 antibody.

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A

site	strain	position	sequence
NFkB	(−)	-2883 to -2871	caGGGAtttaccc
NFkB	(−)	-2768 to -2756	ttgGGGAgaccct
NFkB	(+)	-2750 to -2738	ctGGCAttttcct
NFkB	(+)	-601 to -589	gtttggatTTCCt
NFkB	(+)	-383 to -371	ccaggtgaTTCCa
NFAT	(+)	-2368 to -2350	gacgaGGAAagaattagca
NFAT	(−)	-2175 to -2157	tccaaGGAAagaggagata
NFAT	(−)	-603 to -585	attaaGGAAatccaaacta
NFAT	(−)	-142 to -124	gctgaGGAAattgcacgcg

B

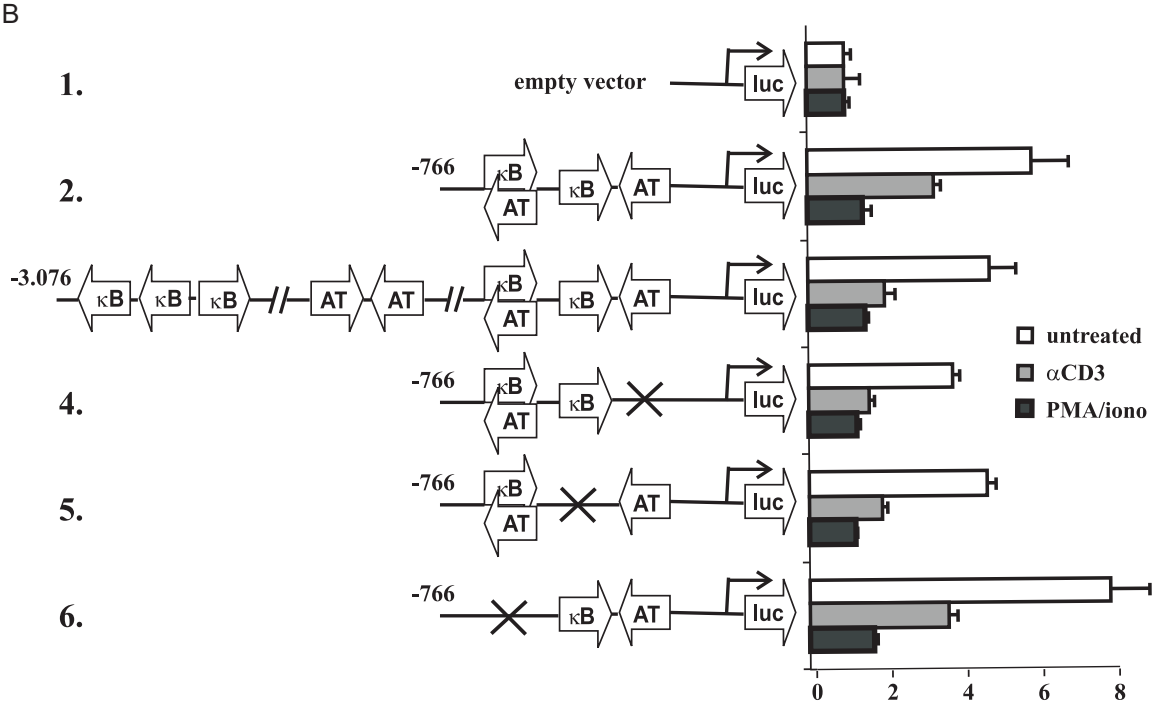

Fig. 4. Relation of NF-κB and NF-AT binding sites in the DR6 promoter to the regulation of DR6 expression in activated Jurkat cells (A) Predicted NF-κB and NF-AT binding sites in the 3 kb region upstream of the transcription initiation site in the DR6 promoter by Genomatix MatInspector software (http://www.genomatix.de/products/MatInspector; Cartharius et al., 2005) (B) Jurkat cells were electroporated with the indicated firefly luciferase reporter vectors with subcloned mutants of the *DR6* promoter (numbered 1–5; schemes in the left panel). The luciferase activity was determined after 48 h of stimulation with either anti-CD3 antibody or PMA/ionomycin treatment.

4. Discussion

Death receptor-6 belongs to the least characterized receptors from the TNFR family but interestingly it is also one of the most conserved TNFR family receptors in vertebrates (Benschop et al., 2009; Bridgham et al., 2001). Previous studies based on data obtained from DR6 knockout mice indicated an important immunoregulatory role for DR6. DR6 apparently participates in dampening immunoreceptor-coupled signaling pathways also via regulating the expression of key signaling molecules such as Th2 cytokines IL-4, -5, -10 and -13, and interestingly DR6 might also affect T-cell migration (Liu et al., 2001, 2002; Schmidt et al., 2003, 2005; Venkataraman et al., 2006; Zhao et al., 2001).

In this communication we analyzed the regulation of DR6 expression in hematopoietic cells. We found that DR6 is not expressed in resting peripheral blood T cells, but it is significantly upregulated in TCR cross-linking-activated T lymphocytes, similarly as some other T-cell response-regulating receptors from this family such as OX40, 4-1BB, GITR or CD30. Though DR6 deficiency in mice enhanced CD4+ but not CD8+ T-cell proliferation (Liu et al., 2001), suggesting that DR6 expression might negatively affect the proliferation or function of mainly CD4+ T cells, we found that both human peripheral blood CD4+ and to an even greater extent CD8+ cells induce the expression of DR6 upon CD3 cross-linking. In contrast to the published data (Zhao et al., 2001), we observed time-dependent increase of *DR6* mRNA expression in negatively selected, activated murine CD4+ T cells. (Suppl. Fig. 1). This apparent discrepancy with their results might be caused by a different CD4+ T cells separation approaches (positive versus negative immunomagnetic preparation). At least in human cells DR6 expression profile mirrors OX40, which is also expressed on both CD4+ and CD8+ T cells upon TCR activation (Baum et al., 1994; Cannons et al., 2001), but mice lacking OX40 or its ligand display, in contrast to DR6-deficient mice, defects in CD4+ T-cell proliferation (Kopf et al., 1999; Pippig et al., 1999). Interestingly, OX40, similarly as DR6, might play a stimula-

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tory role in CD4+ T-cell migration to inflammatory sites (Schmidt et al., 2005; Weinberg et al., 1996).

T-cell activation induces a number of downstream pathways, most notably triggering the activation of the transcription factors NF-κB, NF-AT and AP-1. DR6 expression has been previously shown to be activated in a NF-κB-dependent manner in the prostate cancer cell line LNCaP (Kasof et al., 2001). In agreement with the apparently important role of NF-κB in the regulation of DR6 expression, we also found that T-cell stimulation-triggered DR6 expression critically depends on the activation or activity of NF-κB and also of NF-AT (Fig. 2). Moreover, harassing the proximal TCR signaling feeding into NF-κB or NF-AT activation by inhibiting PI3 kinase and especially Src family kinases also suppressed or blocked DR6 expression. The expression of other TNFR family receptors induced upon T-cell stimulation, such as GITR, OX40 or 4-1BB, is also activated in a NF-κB-dependent manner (Pichler et al., 2008; Tone et al., 2007; Zhan et al., 2008), but in contrast to DR6, NF-AT negatively regulates GITR expression on activated murine T cells (Zhan et al., 2008).

The Jurkat T-cell leukemia cell line has been used to dissect T-cell activation-related responses (Abraham and Weiss, 2004). The stimulation of Jurkat cells via CD3 cross-linking or PMA/ionomycin treatment indeed resulted in a strong upregulation of the early lymphocyte activation marker CD69 as well as of other well-known positive modulators of T-cell signaling from the TNFR family such as GITR, OX40 and 4-1BB. However, in contrast to PBT cells, unstimulated Jurkat cells already expressed significant levels of DR6, and these levels unexpectedly decreased upon cell activation. From profiling TNFR family receptor expression, HVEM and TRAIL receptor DR4 also showed an opposite trend in comparison to activated PBT cells. In agreement with previous reports, HVEM expression dropped in activated PBT cells (Morel et al., 2000) but significantly increased in stimulated Jurkat cells (Suppl. Fig. 3).

Why do primary T cells and Jurkat cells behave so differently in the regard to the regulation of DR6 expression? A possible reason could be related to aberrant and sustained activity of NF-κB in these cells. Constitutively activated NF-κB complexes were also found in the vast majority of childhood acute lymphoblastic leukemias without any subtype restriction (Kordes et al., 2000). The aberrant constitutive activity of NF-κB in Jurkat cells is apparently protein kinase PI3K-Akt/PKB-dependent (Kane et al., 1999) and has been connected with the defect in expression of the phosphoinositide phosphatase PTEN (Shan et al., 2000). In agreement with these findings, DR6 expression could be suppressed by inhibitors of PI3-kinase in non-stimulated Jurkat cells. Moreover, DR6 expression in Jurkat cells may be downregulated also upon cell stimulation via CD3 crosslinking or PMA/ionomycin treatment, most likely by induction of an unknown repressor.

An open question remains the functional consequence of DR6 expression for the proliferation, survival and physiological functions of activated T cells. Though DR6 contains a death domain, and could participate in neuronal cell death (Nikolaev et al., 2009), its T-cell regulatory role might be not related to apoptotic signaling. When we compared death-inducing abilities of overexpressed death receptors (Fas/CD95, TNFR1, TRAIL-R1/DR4, TRAIL-R2/DR5 and DR6) in HEK293 cells in the absence or presence of cycloheximide, DR6 pro-apoptotic signaling was the least efficient one (unpublished data). DR6 deficiency also did not negatively affect AICD of activated mouse T cells (Liu et al., 2001; Zhao et al., 2001), and thus DR6-activated signaling could modify proliferation and/or differentiation of activated T cells. In this matter DR6 resembles another death receptor expressed also in activated T cells – death receptor 3 (DR3), which apparently participates in the regulation of differentiation of Th17 CD4+ T cells (Jones et al., 2011).

The increased expression of DR6 on activated T cells is an essential but not sufficient condition for triggering DR6-dependent signaling and, similarly as for other TNFR family receptors induced on activated T cells, the presence of and activation by the corresponding ligand is required. Recently, a cleaved amino-terminal fragment of β-amyloid precursor protein (*N*-APP) or of its close relative amyloid precursor-like protein-2 (*N*-APLP2) were reported as possible DR6 ligands, which could trigger the DR6-dependent activation of caspases -3 or -6, resulting in the axonal pruning and cell death of spinal neurons from murine embryos (Nikolaev et al., 2009). APLP2 is expressed on human CD19+ B cells and CD14+ monocytes (Suppl. Fig. 4), and thus its cleaved-off *N*-terminal fragment could potentially serve as a ligand for DR6 expressed on activated T cells. However, neither the *N*-APP-Fc or *N*-APLP2-Fc proteins produced in HEK293FT cells nor the His-*N*-APP and His-*N*-APLP2 proteins produced in *E. coli* were able to bind to DR6 and consequently to trigger the usual TNFR receptor-coupled signaling pathways such as caspase-cascade, the activation of NF-κB or the activation of stress kinases from the JNK/SAPK family, which were previously shown to be triggered by DR6 overexpression in some cell lines ((Pan et al., 1998) and data not shown). Possible reasons for this discrepancy with published results could be related to differences between the human and murine systems or to the cell-type dependent interaction between the ligand and DR6, potentially requiring a co-receptor present on embryonic spinal neurons but not expressed by hematopoietic cells. Thus, even though we have shown that the first essential requirement (the presence of DR6 on activated T cells) for a regulatory role of DR6 in T-cell physiology is fulfilled, the nature and function of the DR6 ligand in the hematopoietic system still awaits its discovery.

5. Conclusions

Death receptor 6 remains, despite its teenage age, one of the least characterized receptors from the TNFR superfamily. Data obtained from DR6 knockout mice just point to its apparently regulatory function in proliferation/survival of T and B cells and also of developing neurons (Benschop et al., 2009; Nikolaev et al., 2009). Strikingly, aside from the phenotypic description of DR6-deficient hematopoietic cells no data on the regulation of DR6 expression in these cells are available. Thus we carried out analysis of human peripheral blood cells for DR6 expression and discovered that DR6 expression is significantly but transiently upregulated only in activated both CD4+ and CD8+ T cells in NF-κB and NF-AT dependent manner with a contribution of PI3K-dependent signaling. T-cell leukemia cell line Jurkat already expresses DR6 likely via constitutive activation of PI3K pathway, and in contrast to the primary T cells, CD3 engagement leads to the suppression of DR6 expression at the transcriptional, NF-κB- and NF-AT-dependent level. Our data just provide thus first glue connecting T-cell activation-dependent DR6 expression with its observed role in regulating proliferation and activities of T cells.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2011.03.021.

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ORIGINAL PAPER

Arf and Rho GAP adapter protein ARAP1 participates in the mobilization of TRAIL-R1/DR4 to the plasma membrane

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Abstract TRAIL, a ligand of the TNFα family, induces upon binding to its pro-death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 the apoptosis of cancer cells. Activated receptors incite the formation of the Death-Inducing Signaling Complex followed by the activation of the downstream apoptotic signaling. TRAIL-induced apoptosis is regulated at multiple levels, one of them being the presence and relative number of TRAIL pro- and anti-apoptotic receptors on the cytoplasmic membrane. In a yeast two-hybrid search for proteins that interact with the intracellular part (ICP) of DR4, we picked ARAP1, an adapter protein with ArfGAP and RhoGAP activities. In yeast, DR4(ICP) interacts with the alternatively spliced ARAP1 lacking 11 amino acids from the PH5 domain. Transfected ARAP1 co-precipitates with DR4 and co-localizes with it in the endoplasmic reticulum/Golgi, at the cytoplasmic membrane and in early endosomes of TRAIL-treated cells. ARAP1 knockdown significantly compromises the localization of DR4 at the cell surface of several tumor cell lines and slows down their TRAIL-induced death. ARAP1 overexpressed in HEL cells does not affect their TRAIL-induced apoptosis or the membrane localization of DR4, but it enhances the cell-surface presentation of phosphatidyl serine. Our data indicate that ARAP1 is likely involved in the regulation of the cell-specific trafficking of DR4 and might thus affect the efficacy of TRAIL-induced apoptosis.

Keywords Apoptosis · TRAIL · Interaction · Receptor · Trafficking · Signaling

Abbreviations

TNF	Tumor necrosis factor
TRAIL/Apo2L	TNF-related apoptosis-inducing ligand
Arf	ADP-ribosylation factor small GTPase
ARAP1	ArfGAP, RhoGAP, Ankyrin repeats and pleckstrin homology (PH) domains containing protein
DD	Death domain
DISC	Death-inducing signaling complex
ER	Endoplasmic reticulum
GAP	GTPase-activating protein
IAP	Inhibitor of apoptosis
MFI	Median fluorescence intensity
MMP	Mitochondrial membrane permeabilization
PARP	Poly-ADP ribose polymerase
SiRNA	Small inhibitory RNA
TGN	Trans-Golgi network

Introduction

TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of the tumor necrosis factor (TNF) family and is mainly expressed on the surface of hematopoietic cells (B-lymphocytes, antigen-activated T cells, NK cells and monocytes), though its mRNA has also been detected in many other tissues such as spleen, lung and prostate [1, 2].

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TRAIL preferentially induces the apoptosis of malignant cells rather than normal cells [3, 4] and interacts with five different receptors: apoptosis-inducing receptors DR4/TRAIL-R1 and DR5/TRAIL-R2, decoy receptors DcR1/TRAIL-R3 without the intracellular part and DcR2/TRAIL-R4 with a truncated death domain, and osteoprogeterin (OPG) [5–9]. Death receptors DR4 and DR5 are expressed on most, if not all, normal somatic cells; nevertheless, on these cells their engagement with TRAIL usually does not trigger apoptosis [1, 5, 6]. The combination of the lower expression of pro-apoptotic receptors and the increased expression of decoy receptors and of intracellular anti-apoptotic proteins such as FLIP or Mcl-1 provides protection for healthy human cells against TRAIL-induced apoptosis. However, the sensitivity of normal human cells to TRAIL-induced apoptosis is greatly enhanced by their transformation, viral infection, or other pathological processes [10].

The intracellular part of the death receptors contains an approximately 80 amino acid long, conserved domain called the death domain (DD) [11]. This structural motif is essential for the formation of the Death-Inducing Signaling Complex (DISC) and the induction of apoptosis [12]. The basic TRAIL DISC is composed of the ligand, the receptor(s), the adapter protein FADD, and the initiator pro-caspase-8 or -10 and is formed shortly after TRAIL binding to its receptors [13]. Pro-caspase-8, recruited to the DISC via FADD, is rapidly activated by auto-proteolytic cleavage; its activated pro-domains persist in the DISC and cleave downstream effector proteins [14]. Active caspase-8 can directly activate the executioner caspases (-3, -6, -7) in so-called type I signaling. Caspase-8-mediated cleavage of Bid is required for the translocation of truncated Bid (tBid) to the mitochondria and the activation of type II mitochondrial apoptotic signaling [15, 16]. Besides the expression and cell-surface localization of pro- and anti-apoptotic TRAIL receptors, TRAIL-induced apoptosis of human tumor cells is regulated by the inhibition of DISC assembly, ineffective downstream signal transduction (Bid processing and translocation, Bax/Bak activation), or by the suppression of the amplification loop at or downstream of the mitochondria (inhibition of MMP or activated effector caspases via IAPs) [10, 17, 18].

The first and essential condition for the productive initiation of TRAIL-induced apoptosis is the expression and proper cellular localization of the proapoptotic TRAIL receptors DR4 and DR5. They are predominantly localized at the cell surface and in the ER/TGN system [19]. A similar pattern of localization was also described for TNFR1 and CD95 [20–22]. Their transport from the TGN to the cellular membrane is apparently mediated by cargo-transport vesicles composed of Arf GTP-binding proteins, coat proteins such as COPI or clathrin, GGA and tetrameric adaptor proteins (APs), and transported receptors [23]. Among Arf proteins, Arf1 is required for the recruitment of coat proteins from the cytosol onto the membranes of the TGN [24]. Arf-protein cargo-related activity is regulated by a family of Arf GAP proteins such as ArfGAP1 or AZAP proteins that activate GTP hydrolysis and thus induce Arf dissociation from the coated vehicles [25]. Arf GAPs, however, not only serve as catalysts of Arf release and recycling, but they also represent a group of coat proteins that can interact with cargo proteins (e.g., transported receptors). Arf GAP1 interacts with the ERD2 receptor via its C-terminal domain, while ACAP1, a member of the AZAP family, binds to the cytoplasmic tail of the human transferrin receptor and promotes its recycling [26, 27].

Here we present evidence that ARAP1/centaurin δ2, a member of the AZAP family [28], can interact with DR4 both in yeast and in mammalian cells. siRNA-mediated downregulation of ARAP1 markedly and specifically reduced the cell-surface localization of DR4 in several human cell lines and attenuated the kinetics of their TRAIL-induced apoptosis. Our data thus indicate that ARAP1 could be involved in the regulation of DR4 cellular trafficking.

Materials and methods

Cells, reagents and antibodies

Human immortalized keratinocytes NCTC were provided by Dr. L. LoMuzio, MG-63 and Saos-2 by Dr. P. Hozak, human colon carcinoma cells HCT-116, DLD-1, HT29 and the erythroleukemia cell line HEL (human erythroleukemia) were purchased from ATCC and HEK293FT from Invitrogen. Human recombinant TRAIL (amino acids 95–281) was prepared in our laboratory as described previously [29]. Mouse monoclonal antibodies against the extracellular parts of DR4, DR5, DR6 and against the C-terminal part of ARAP1 (amino acids 1,084 to 1,372) were prepared in our laboratory. DR4 blocking antibody DR4-02 was purchased from Exbio. The following commercially available antibodies were obtained from the indicated suppliers: mouse monoclonal anti-Myc tag (Roche Molecular Biochemicals), rabbit polyclonal anti-caspase-8 (BD Biosciences), rabbit polyclonal anti-DR4, DR5, Bid, PARP and Rab5A (Santa Cruz). Rabbit polyclonal anti-ARAP1 antibody was kindly provided by Dr. P.A. Randazzo. Anti-TRAIL receptor-1 to -4 flow cytometry set and rabbit polyclonal anti-DR4 and DR5 antibodies were purchased from Alexis. Secondary Alexa-488- and Alexa-594-conjugated goat anti-rabbit IgG and Alexa-488- and -594-conjugated goat anti-mouse IgG antibodies were supplied by Molecular Probes. FITC-conjugated anti-M30 antibody was purchased from Roche

Molecular Biochemicals. Mouse monoclonal anti-CD43 and anti-β1-integrin antibodies were kindly provided by Dr. V. Horejsi and anti-α-tubulin antibody by Dr. P. Draber. Anti-DR4, DR5, DR6 and CD43 (MEM-59) antibodies used for immunoprecipitations were coupled to activated CNBr Sepharose (GE Healthcare) according to the manufacturer's protocol.

Plasmids

KIAA0782 plasmid encoding ARAP1 cDNA was supplied by Kazusa DNA Research Institute (Chiba, Japan). The cDNA was subcloned into the *Bam*HI and *Bgl*II sites of pKMyc-C3 (modified pCDNA3 with a Myc tag upstream of the MCS). Full-length ARAP1 (containing the SAM domain and lacking exon 30) was prepared by PCR-assisted subcloning from NCTC cDNA. pCDNA3-based DR6 and CD43 expression plasmids were prepared in our laboratory. pCR-DR4 and pCR-DR5 were kindly provided by Dr. J. Tschopp. Deletion mutants of DR4 were prepared by PCR using the primers shown in the Supplementary Table 1 and subcloned into the *Eco*RI and *Bam*HI sites of pCR3 plasmid. DR4-ICP was prepared by PCR (for primers—see Suppl. Table 1) and cloned into the *Eco*RI and *Bam*HI sites of pGBKT7. All PCR products were verified by sequencing.

Two-hybrid cDNA library screening

The yeast two-hybrid screening for DR4(ICP)-interacting proteins was performed using the Matchmaker 3 system (Clontech), according to the manufacturer's protocol. AH109 yeasts transformed with pGBKT7-DR4-ICP were mated with Y187 cells containing HA-tagged human cDNA libraries (leukocyte or bone marrow) cloned into the pACT2 plasmid (Clontech). Upon auxotrophic selection on SD/agar plates lacking tryptophan, leucine, adenine, and histidine (SD-WLAH), the surviving colonies were transferred to SD/agar plates lacking tryptophan, leucine (SD-WL) plates, and plates containing 5-brome-4-chloro-3-indolyl-β-D-galactosid (X-Gal). Blue colonies were then grown in the selective WLAH medium and analyzed by Western blotting with anti-HA antibody. Clones with a molecular weight above 35 kDa (i.e., with an insert longer than 80–100 amino acids) were sequenced and checked for the specificity of their interaction with the bait.

Cell culture and transfection

HEK293FT, HCT116, MG-63 and NCTC cells were cultured in Dublecco's modified Eagle's medium (DMEM), and Saos-2 in RPMI medium containing 10% fetal calf serum (FCS). HEL cells grown in RPMI medium with 10% FCS were transfected with the pKMyc-C3 ARAP1 (Δexon 30) expression plasmid or with the empty vector (mock transfection), and ARAP1-expressing clones were selected using geneticin G418 (Alexis). Transfection of all the cell lines was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Immunoprecipitation and Western blotting

Cells for immunoprecipitation were washed twice with ice-cold PBS and lysed on ice in 1 ml of the lysis buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 1 mM sodium orthovanadate, and protease inhibitors (Complete, Roche), pH 7.5. Cell lysates were centrifuged at 4°C and 13,000 rpm for 30 min, and the supernatants were immunoprecipitated with the CNBr Sepharose-coupled antibodies at 4°C for 4 h. The immunoprecipitated complexes were washed five times with the lysis buffer and eluted from the immunosorbent with SDS sample buffer. Proteins in the immunocomplexes or the cell lysates were separated by SDS PAGE and transferred onto nitrocellulose membranes (Amersham). Membranes were blocked with 5% nonfat milk in PBS containing 0.05% Tween (PBS/Tween), incubated with specific primary antibodies and, after washes, with the appropriate peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (BioRad). After three washes with PBS/Tween, the bound secondary antibodies were visualized by enhanced chemoluminescence (ECL, Pierce).

Confocal microscopy

NCTC cells were cultured on coverslips in a 24-well culture dish, transfected with the corresponding plasmids and 24 h later treated with 200 ng/ml TRAIL. At given time points after adding TRAIL, the cells were washed with ice-cold PBS, fixed with methanol at -20°C for 5 min and permeabilized with acetone. Fixed cells were incubated in blocking buffer (PBS with 1% bovine serum albumin) for 20 min, then with the primary antibodies for an additional 30 min, washed three times with PBS, and further incubated with the appropriate secondary antibodies at room temperature for 30 min. The coverslips were mounted on slides using Mowiol mounting medium and analyzed by confocal microscopy (Leica TCS SP confocal laser scanning microscope).

Down-regulation of ARAP1 expression

A mix of or individual ARAP1 siRNAs (siGenome, Dharmacon) or control Luciferase siRNAs CAAGCUGA

CCCUGAAGUUCdTdT (Dharmacon) were used for Lipofectamine RNAiMax (Invitrogen)-mediated transfection of the analyzed cell lines. The sequences of selected ARAP1 siRNA were: oligo #1 GAAUAAGCUGUACGUGGCCdTdT, oligo #2 CUUCGUGGCUUCAAGAAU AdTdT. Cells were cultured in 24-well culture dishes and transfected at 40–50% confluence. Fifty-two hours later, ARAP1 downregulation in siARAP1 transfected cells was confirmed by Western blotting, and the cells were used in follow-up experiments (analysis of the surface expression of receptors, TRAIL-induced apoptosis, etc.).

Flow cytometry and apoptosis assays

Adherent cells were released by treatment with PBS containing 10 mM EDTA and, after washing with ice-cold PBS, were incubated in blocking solution (PBS with 20% human serum, 1% gelatin and 0.1% azide) on ice for 15 min. Cells were then washed once with wash buffer (PBS containing 1% gelatine and 0.1% azide), incubated with the appropriate primary monoclonal antibodies, washed twice with ice-cold wash buffer and finally incubated with the secondary goat anti-mouse antibody coupled to phycoerythrin (IgG1-PE) (SouthernBiotech). All incubations were performed on ice. After two final washes, the surface expression of the receptors on living cells (Hoechst 33258 negative) was analyzed using a LSRII flow cytometer (BD Biosciences). M30 Cytodeath assay (Roche) of caspase-3-cleaved cytokeratine 18 or Annexin V-FITC/Hoechst 33258 (Alexis) staining were used for the assessment and quantification of TRAIL-induced apoptosis according to the manufacturer's protocols. M30-FITC or Annexin V-FITC stained cells were analyzed by flow cytometry using a LSRII flow cytometer.

Results

Rho and Arf GAP adapter protein ARAP1 lacking exon 30 specifically interacts with the intracellular part of DR4/TRAIL-R1

Activation of TRAIL-induced apoptosis ultimately depends on the presence and status of TRAIL's proapoptotic receptors (cell surface expression, mutations, competition of decoy receptors) and on the kinetics and efficacy of DISC formation/activation (type I vs. type II, activation inhibitors such as FLIP) [31, 32]. Most of these critical regulatory events are mediated via protein–protein interactions. In order to search for new proteins that interact with TRAIL-R1/DR4 and could affect its signaling, we performed a yeast two-hybrid (Y2H) screening of several cDNA expression libraries using the intracellular part of DR4 (ICP, amino acids 246–445) as a bait. Two fusion proteins specifically interacting with DR4(ICP) contained a C-terminal part of ARAP1 (centaurin δ2), an adapter protein with multiple domains and Arf and Rho GAP activities ([28] and Fig. 1a). A comparison of the interacting C-terminal part of ARAP1 (ARAP1-C) with the Genebank database revealed that this ARAP1 clone does not contain the very short exon 30 (translated to amino acids 1,319–1,329) that maps into the fifth pleckstrin homology (PH) domain (Fig. 1a). Interaction analysis in yeasts uncovered that the presence of exon 30 in the ARAP1 C-terminus abolished its interaction with the intracellular part of DR4 (Fig. 1b). Similarly as the C-terminal part of ARAP1 (FL), the corresponding C-termini of its relatives ARAP2 and ARAP3 also did not bind to the intracellular part of DR4 in Y2H (not shown).

The specific interaction of the intracellular part of DR4 with the exon 30-less splice variant of ARAP1 posed the question of the relevance of ARAP1Δexon30 expression in human cell lines. We therefore employed quantitative real-time PCR to analyze cDNAs from several human tumor-derived cell lines as well as from different primary cells for the relative expression of ARAP1 and ARAP1Δexon30 using exon-30 flanking primers. The data from this analysis showed that the Δexon 30-less splice variant is the predominantly expressed form in a majority of the tested cell lines, most notably in NCTC, DLD-1 and HEK293 cells as well as in primary fibroblasts, colon epithelial and T cells (Suppl. Fig. 1a). The specificity of the RT-qPCR analysis of ARAP1 splice variant expression was confirmed by ApaI cleavage of the PCR-amplified 3′-end of ARAP1. The *Apa*I cleavage site resides in exon 30, and only a minor part of the ARAP1 3′ end amplified from NCTC cDNA was cleaved by this enzyme, verifying that the major ARAP1 splice variant in NCTC cells does not contain exon 30 (Suppl. Fig. 1b).

ARAP1 interacts and co-localizes with DR4 in human cells

Uncovering ARAP1 as a new DR4-interacting protein with a potential regulatory function prompted us to examine their interaction in human cells as well as the possible functional consequences of this interaction. First of all we examined whether transiently expressed ARAP1 C-terminus (ARAP1-CΔexon30) and ARAP1Δexon30 (ARAP1 full length lacking exon30) can interact with co-transfected DR4 or other death (DR5 and DR6) and control (CD43) receptors in HEK293FT cells. Myc-tagged ARAP1-CΔexon30 co-immunoprecipitated with DR4 and, to lesser extent, DR5 (Fig. 2b, lanes 2 and 8) while it did not

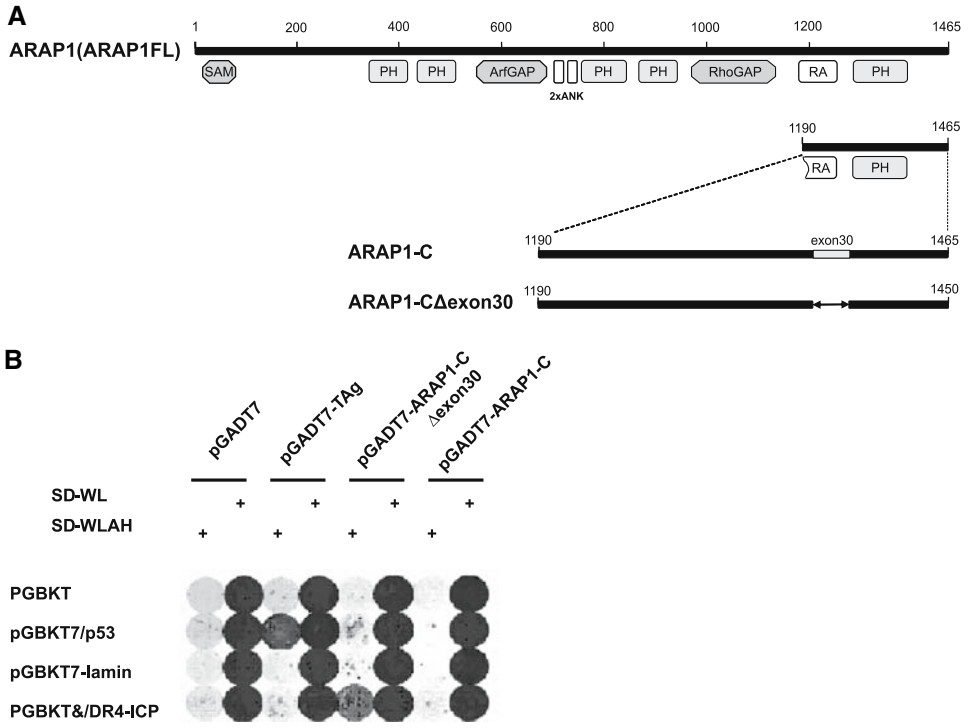

Fig. 1 ARAP1 (Δexon30) associates with DR4(ICP) in yeasts. (**a**) Schematic illustration of human ARAP1 (ARAP1 transcription variant 3, NM_001040118.1). Full-length ARAP1 (1465 amino acids) contains the N-terminal sterile α motif (SAM) domain, Arf GAP and Rho GAP domains, 5 pleckstrin homology domains (PH), two ankyrin repeats (ANK) and a Ras-associating domain (RA). The C-terminus of ARAP1, ARAP1-CΔexon30 (exon 30-lacking splice variant), was pulled out from the human leukocyte cDNA library by yeast two hybrid screening for DR4(ICP)-interacting proteins. (**b**) Intracellular part of DR4, DR4(ICP), interacts in yeasts only with the ARAP1-CΔexon30 variant. AH109 yeasts containing pGBKT7 bait plasmids were mated with Y187 yeasts transformed with the appropriate pGADT7 plasmids. Mated yeasts were grown first on a plate with the mating medium (SD-WL—every second column), then transferred onto a plate with the selection medium (SD-WLAH) to test the interaction (every first column). The mating couple pGBKT7-p53 and pGADT7-Tag was used as a positive control for mating and pGBKT7-lamin was used as a negative control

interact with DR6 or CD43 (Fig. 2b, lanes 10 and 12). Ectopically expressed full-length ARAP1Δexon30 co-immunoprecipitated with DR4 as well, albeit less efficiently than its C-terminal part (Fig. 2b, lane 14).

For mapping the ARAP1-interacting region in the intracellular part of DR4, we prepared a set of its deletion mutants (Fig. 2a) and analyzed their interaction with the C-terminal part of ARAP1. Removal of last 22 amino acids from the DR4 C-terminus (DR4-ΔC) compromised its interaction with ARAP1-CΔexon30 (Fig. 2b, compare lanes 2 and 3). A DR4 deletion mutant lacking the death domain and C-terminal extension (DR4-ΔDDC) failed to interact with ARAP1-CΔexon30 indicating a requirement for the death domain for DR4-ARAP1 interaction (Fig. 2b, lane 4). In our first set of experiments, we also prepared a DR4 deletion mutant that, in addition to the membrane proximal part, also contained the first α-helix and turn region from DR4 DD (DR4ΔxDDC). The strong interaction of this DR4 mutant with ARAP1-CΔexon30 suggested that this part of the DR4 DD could contain the ARAP1 interaction motif (Fig. 2b, lane 6). A comparison of first α-helices in the death domains of several human death receptors revealed that the predicted turn region between the first and second α-helix (C-terminus of the DR4ΔxDDC mutant) contains, in DR4 and DR5, conserved phenylalanine and aspartate (Suppl. Fig. 2a). Their substitution with the corresponding amino acids from TNFR1 (proline and leucine) in mutant DR4-FD/PL led to the severe suppression of ARAP1-CΔexon30 co-immunoprecipitation with DR4 (Fig. 2b, lane 7).

In comparison with ARAP1-CΔexon30, the interaction of full length ARAP1Δexon30 with DR4 was relatively weak. Thus, it was not very surprising that we were unable to co-immunoprecipitate the endogenous proteins from the whole cell lysates of several analyzed human cell lines (not shown). Only upon TRAIL treatment of NCTC cells did we observe the co-immunoprecipitation of the apparently processed form of ARAP1 with endogenous DR4 (Fig. 2c). The appearance of the ARAP1 processed form and its co-immunoprecipitation with DR4 was dependent on caspase activity (Suppl. Fig. 2b). Thus, it is likely that the interaction of the full-length (transfected or endogenous) proteins with one another is either weak or transient and

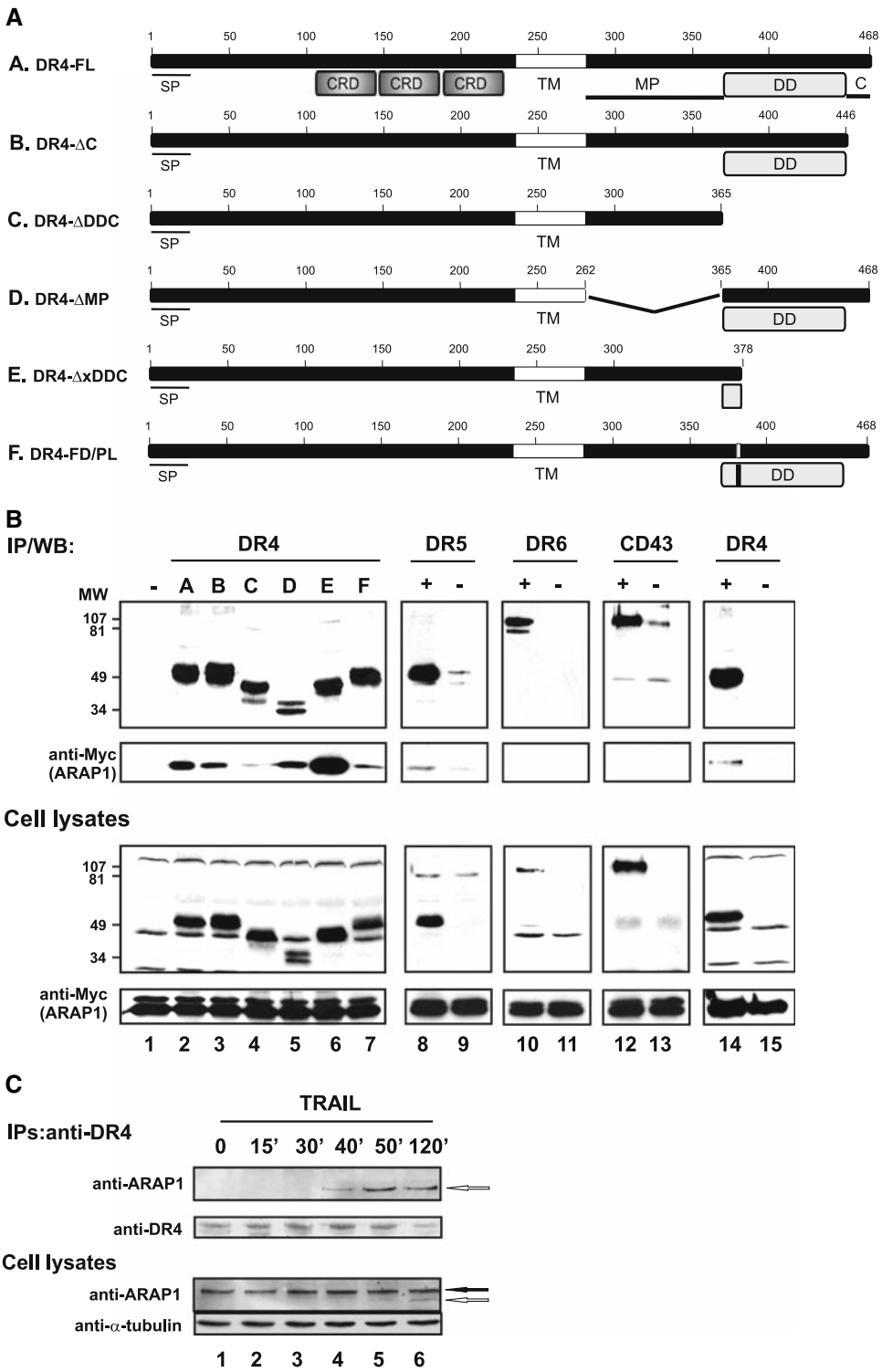

Fig. 2 ARAP1 interacts with DR4 in human cells. (**a**) Graphical representation of DR4 mutants (SP, Signal Peptide; CRD, Cysteine Rich Domain; TM, transmembrane domain; MP, membrane proximal region; DD, Death Domain; C, C terminus). (**b**) HEK293FT were transfected with pKMycARAP1-CΔexon30 either alone (lanes 1, 9, 11, 13) or together with the expression plasmids for DR4FL and mutants (A-F)—lanes 2–7, DR5 (lane 8), DR6 (lane 10), CD43 (lane 12); or with plasmid expressing ARAP1Δexon30 alone (lane 15) or together with DR4FL (lane 14). Twenty-four hours after transfection, the cells were lysed and the lysates were immunoprecipitated with the corresponding antibodies (anti-DR4, -DR5, -DR6 or -CD43) coupled to CNBr Sepharose. Immunoprecipitated complexes and the cell lysates were analyzed by Western blotting with the corresponding antibodies (WB, blotting antibody; IP, immunoprecipitating antibody). (**c**) NCTC cells were treated with TRAIL (200 ng/ml) and at the time points indicated in the figure harvested and lysed; DR4 complexes were immunoprecipitated with anti-DR4 Sepharose. Western blots of the immunoprecipitates and cell lysates were stained with anti-DR4, anti-ARAP1 or anti-a-tubulin (loading control). Arrows depict the position of ARAP1 (filled) or its processed form (empty)

could be stabilized by removing the inhibitory/destabilizing sequences.

An obvious requirement for the interaction of two proteins (or protein complexes) is their proximity within the cellular compartments. We co-transfected ARAP1Δexon30 together with DR4 into NCTC cells and analyzed their relative localization in untreated and TRAIL-treated cells (Fig. 3). In untreated cells transfected DR4 was localized at the membrane and in the ER/Golgi compartment surrounding the nuclei. ARAP1 was positioned in the ER/Golgi compartment in agreement with previously published data, but a significant fraction of

ARAP1 was also localized at the cellular membrane. At both sites ARAP1 apparently co-localized with DR4 (Fig 3a, 0′, arrowheads). Upon TRAIL treatment, both ARAP1 and DR4 changed their original positions and subsequently co-localized in early endosomes as detected with anti-Rab5 antibody (Fig. 3a, b, c, 60′, arrows).

Overexpression of ARAP1 led to enhanced phosphatidyl serine exposure but not to the acceleration of TRAIL-induced apoptosis in DR4-positive HEL cells

ARAP1 via its association with DR4 could affect either DR4-induced signaling or localization. Initially, we examined the effect of transient ARAP1 overexpression on DR4 membrane localization and TRAIL-induced apoptosis in HEK293FT cells. Neither DR4 surface expression nor TRAIL-induced apoptosis was significantly affected by overexpressed ARAP1 (not shown). However, HEK293FT cells express both TRAIL receptors DR4 and DR5, and thus an analysis of ARAP1's effect on DR4-induced signaling is affected by concurrent DR5 signaling.

We analyzed the cell surface expression of TRAIL receptors in a panel of human cell lines and discovered that the human erythroleukemia cell line HEL uniquely expresses only DR4 (Fig. 4a). We stably transfected HEL cells (which express low levels of ARAP1) either with ARAP1Δexon30 expression plasmid or with the empty vector. In selected mock transfected (2E, 3E) and

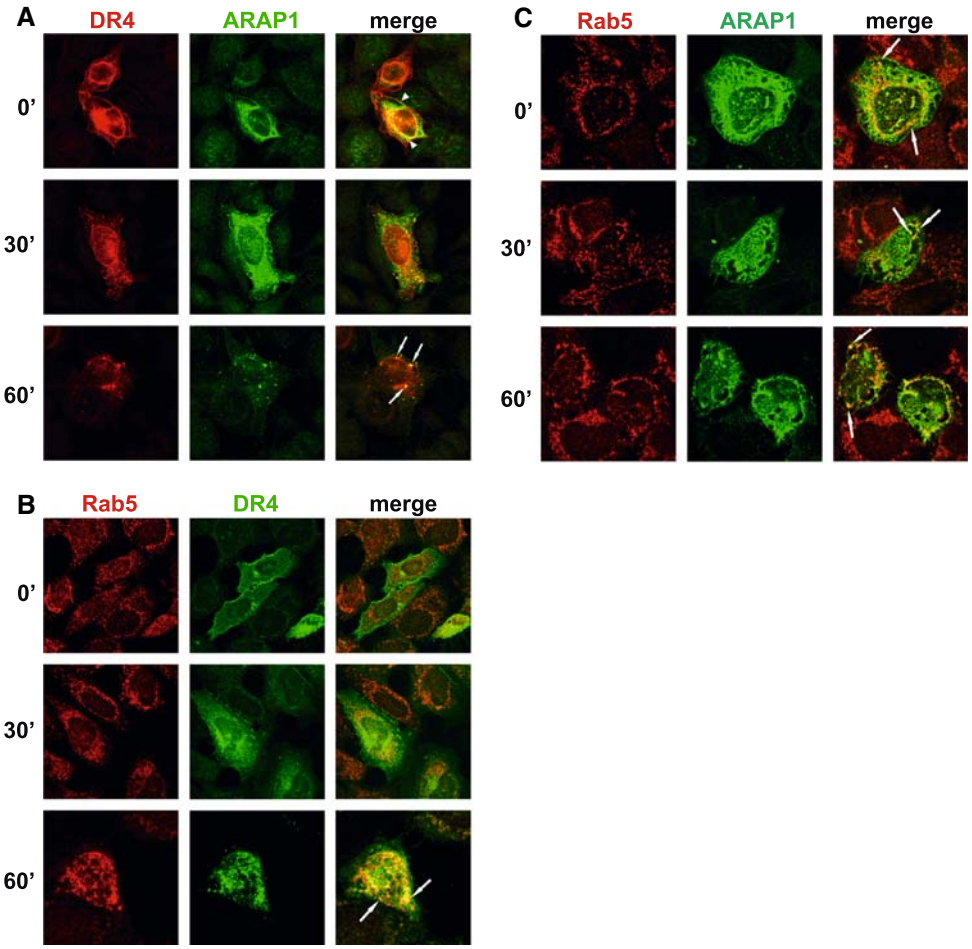

Fig. 3 ARAP1 co-localizes with DR4 at the cell membrane and in early endosomes. (**a**) NCTC cells grown in 24-well plates on coverslips were transfected with pKMyc-ARAP1Δexon30 and pCR-DR4FL. Twenty-four hours after transfection, the cells were treated with TRAIL (200 ng/ml) for 0, 30 or 60 min and fixed with methanol. Fixed cells were stained with rabbit polyclonal anti-DR4 (red) and mouse monoclonal anti-ARAP1 (green) antibodies and with appropriate secondary antibodies Alexa-594 anti-rabbit IgG and Alexa488 anti-mouse IgG. Arrowheads indicate ARAP1-DR4 co-localization at the plasma and intracellular membranes, while arrows show the co-localization of ARAP1 with DR4 in early endosomes after 60 min of TRAIL treatment. (**b**) and (**c**) NCTC cells were transfected either with pCR-DR4FL alone (**b**) or with pKMyc-ARAP1 Δexon30 alone (**c**), treated and fixed as above. The fixed cells were stained either with mouse monoclonal DR4 (**b**) or ARAP1 (**c**) antibodies in combination with rabbit polyclonal Rab5A antibody and with secondary antibodies Alexa488 anti-mouse IgG and Alexa594 anti-rabbit IgG. Arrows indicate the co-localization of either DR4 (green) with Rab5 (red) (**b**) or ARAP1 (green) with Rab5 (red) (**c**) in early endosomes

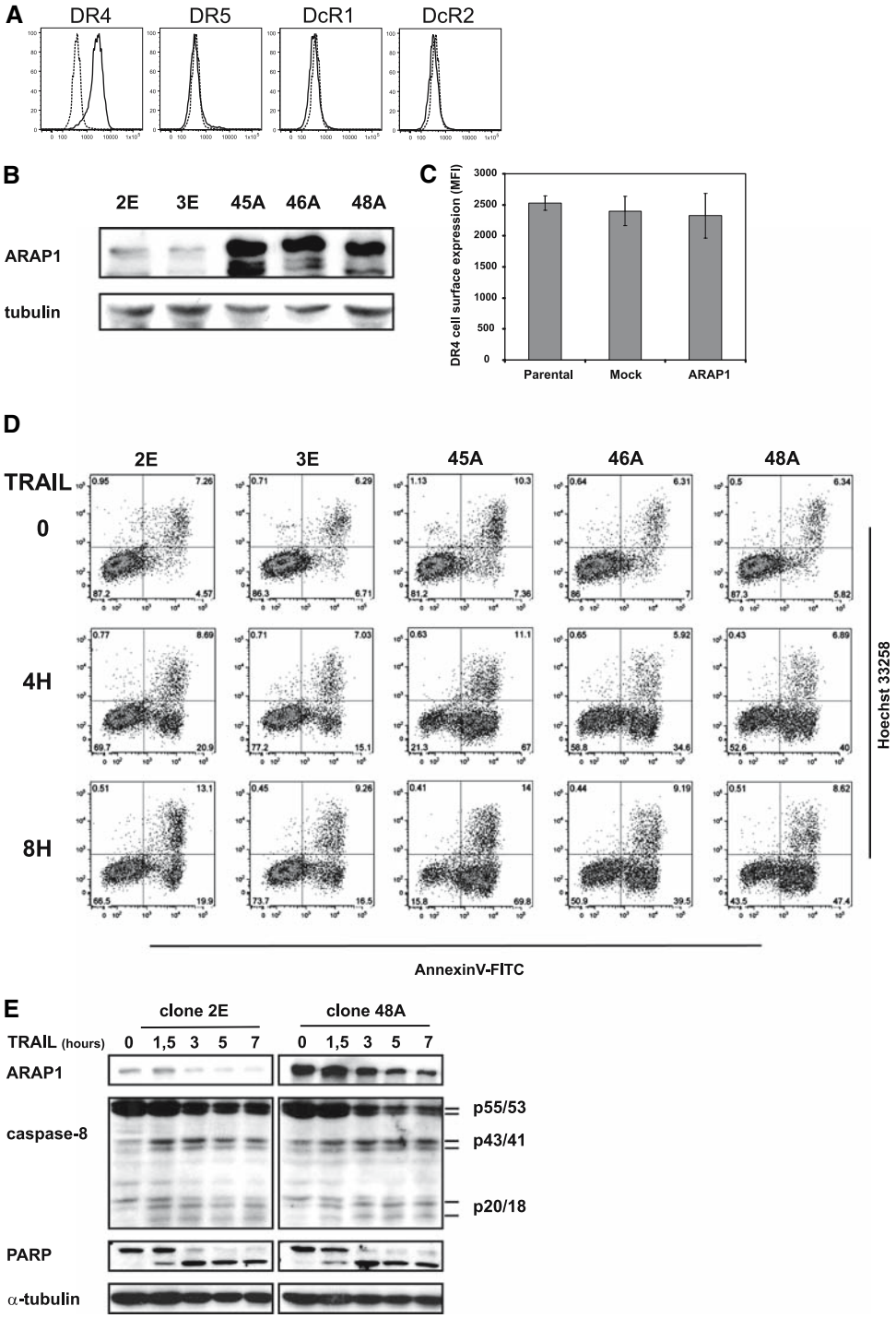

Fig. 4 ARAP1 enhances phosphatidyl serine exposure during TRAIL-induced apoptosis of HEL cells. (**a**) HEL cells were stained with anti-TRAIL receptor antibodies, and the cell surface expression of TRAIL receptors was analyzed by flow cytometry. (**b**) HEL cells were stably transfected either with an ARAP1-expressing plasmid or with the empty vector. Two control clones with the empty vector (2E, 3E) and three HEL clones expressing ARAP1 (45A, 46A, 48A) were analyzed by Western blotting using mouse monoclonal anti-ARAP1 antibody and anti-α-tubulin antibody as a loading control. HEL cells express a low level of endogenous ARAP1 (weak band in lines 2E and 3E). (**c**) Cell surface expression of DR4 in parental HEL cells, mock-transfected (mock) and ARAP1 clones. The average mean and standard deviation of DR4 cell surface expression represented as MFI of 4 independent analyses of parental HEL cells and 4 mock- and 5 ARAP1-transfected HEL clones are shown. (**d**) Selected clones were treated with TRAIL (200 ng/ml) for 4 or 8 h. After the treatment the cells were washed with PBS, stained with FITC-conjugated Annexin V and Hoechst 33258 and analyzed by flow cytometry. The values in the dot plots indicate the percentage of Annexin V- and/or Hoechst-positive cells. (**e**) Total cell lysates from clone 2E and clone 48A taken at the given time points were analyzed by Western blotting using the corresponding antibodies (α-tubulin antibody was used as a loading control)

ARAP1 expressing HEL clones (45A, 46A, 48A) (Fig. 4b), we analyzed the effect of ARAP1 expression on DR4 cell surface expression and TRAIL-induced apoptosis. Overexpressed ARAP1 did not significantly affect the cell surface expression of DR4 in selected HEL clones (Fig. 4b). An initial comparison of TRAIL-induced apoptosis between mock-transfected and ARAP1-expressing cells revealed that phosphatidyl serine exposure detected by Annexin V-FITC is significantly enhanced in ARAP1-expressing HEL clones (Fig. 4c). This observation suggested that ARAP1 overexpression could accelerate TRAIL-induced apoptosis. Nonetheless, biochemical analysis did not confirm these indications, and TRAIL-induced caspase-8 activation or PARP cleavage were comparable in mock-transfected and ARAP1 expressing cells (Fig. 4d). Thus, ARAP1 apparently affected only some cytoplasmic membrane-related and apoptosis-connected changes, such as phosphatidyl serine (PS) exposure,

without having a significant impact on downstream apoptotic signaling.

ARAP1 participates in the regulation of DR4 localization at the cytoplasmic membrane

From confocal microscopy showing ARAP1-DR4 co-localization at the plasma membrane (Fig. 3) and an apparent role for ARAP1 in enhancing PS exposure at the cell membrane (Fig. 4c), we hypothesized that ARAP1 could influence membrane-related events in DR4 signaling. As ARAP1 overexpression did not significantly affect TRAIL-induced apoptosis or DR4 plasma membrane expression, we undertook an opposite approach. Using siRNA we downregulated ARAP1 expression in a panel of human cell lines (Fig. 5a). ARAP1 knockdown in these cells did not affect total DR4 mRNA (not shown) and protein levels (Fig. 5a) but, in some of them (NCTC, Saos-2 and MG-63), did significantly suppress DR4 and, to a lesser extent, DR5 cell surface expression. The cell surface localization of other death receptors (TNFR1 or DR6) or CD29/β1integrin was not affected (Fig. 5b). Interestingly, ARAP1 knockdown in colon cancer-derived HCT116 or DLD1 cells did not reduce DR4 plasma membrane localization at all (Fig. 5b and not shown). ARAP1 siRNA-mediated cell surface downregulation of DR4 in tested cells was statistically significant (Fig. 5c and Suppl. Fig. 3) and was reproducibly observed with two independent ARAP1 siRNAs (Fig. 5d).

Downregulation of ARAP1 by siRNA decelerates TRAIL-induced apoptosis

Next, we asked whether and how ARAP1 siRNA-mediated downregulation of DR4 from the cell surface would affect TRAIL-induced apoptosis in these cells. NCTC (Fig. 6a) and MG-63 (Suppl. Fig. 4) cells transfected either with ARAP1 or with control (luciferase) siRNAs were treated with TRAIL. The kinetics of TRAIL-induced apoptosis was analyzed by M30-FITC staining (caspase-3-mediated cleavage of cytokeratin 18). In contrast to the control cells, the activation of caspases in NCTC cells with downregulated ARAP1 was significantly attenuated—45 min after adding TRAIL, only 24.5% of siARAP1-treated cells were M30-positive, compare to 55.6% of siLuciferase-treated cells (Fig. 5a, 45′). Two hours after the treatment, both ARAP1 and luciferase siRNA-transfected cells displayed comparable M30 staining (not shown). MG-63 cells also behaved similarly, though with less pronounced differences between control and ARAP1 siRNA transfected cells (Suppl. Fig. 4). To exclude the possibility of an off-target effect influencing the kinetics of apoptosis in ARAP1 siRNA-treated cells, we blocked DR4 signaling in NCTC and MG-63 cells with DR4-blocking mAb and examined the effect on the kinetics of TRAIL-induced apoptosis. In comparison with the control block of DR6, in both cell lines the blocking of DR4 signaling slowed down the kinetics of TRAIL-induced cell death to a similar extent as did ARAP1 siRNA (Fig. 6a and Suppl. Fig. 4).

In addition to the quantification of TRAIL-mediated apoptosis, we also analyzed the activation of TRAIL-induced signaling in NCTC cells by Western blotting (Fig. 5b). NCTC cells with downregulated ARAP1 (siARAP1, lanes 7–12) did not change their total level of DR4 (compare the DR4 signal in siLuciferase with siARAP1), but they showed a marked diminution of caspase-8 processing and Bid or PARP cleavage.

In conclusion, our data show that in some cells, ARAP1 could participate in the regulation of DR4 cell surface localization and thus could affect the kinetics and efficacy of TRAIL-induced apoptosis via this receptor.

Discussion

The foremost requirements for the initial steps of TRAIL-induced apoptosis are the cell surface availability of its proapoptototic receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 and productive DISC formation. In addition to DISC-associated proteins, such as procaspases-8/-10, FADD and FLIPs, several other proteins have been reported to interact with the intracellular parts of DR4/DR5 and affect TRAIL-induced signaling. RIP1 co-immunoprecipitates with DR4 and is likely involved in the TRAIL-induced activation of Jun kinases and necrotic cell death [33, 34]. Different splice variants of IG20 interact with the intracellular parts of TRAIL receptors and either enhance or suppress TRAIL apoptotic signaling [35, 36]. Our effort to uncover novel DR4-interacting proteins that could regulate or participate in DR4-dependent signaling resulted in the discovery of the Arf and Rho GAP protein ARAP1. ARAP1 specifically interacts with the intracellular part (ICP) of DR4 both in yeast and in human cells. In contrast to the published sequence of ARAP1, we have found that the C-terminal, a DR4-interacting region comprising part of RA and the last PH domain, contains an alternative exon 30 within the fifth PH domain. At least in yeast, DR4(ICP) interacts only with this exonless variant of ARAP1. As we have shown in Suppl. Fig. 1, this ARAP1 (Δexon 30) is preferentially expressed in the majority of the tested human cell lines and primary cells. Exon 30 codes for 11 amino acids and is localized at the C-terminus of the 3-phosphoinositide binding motif [28], and

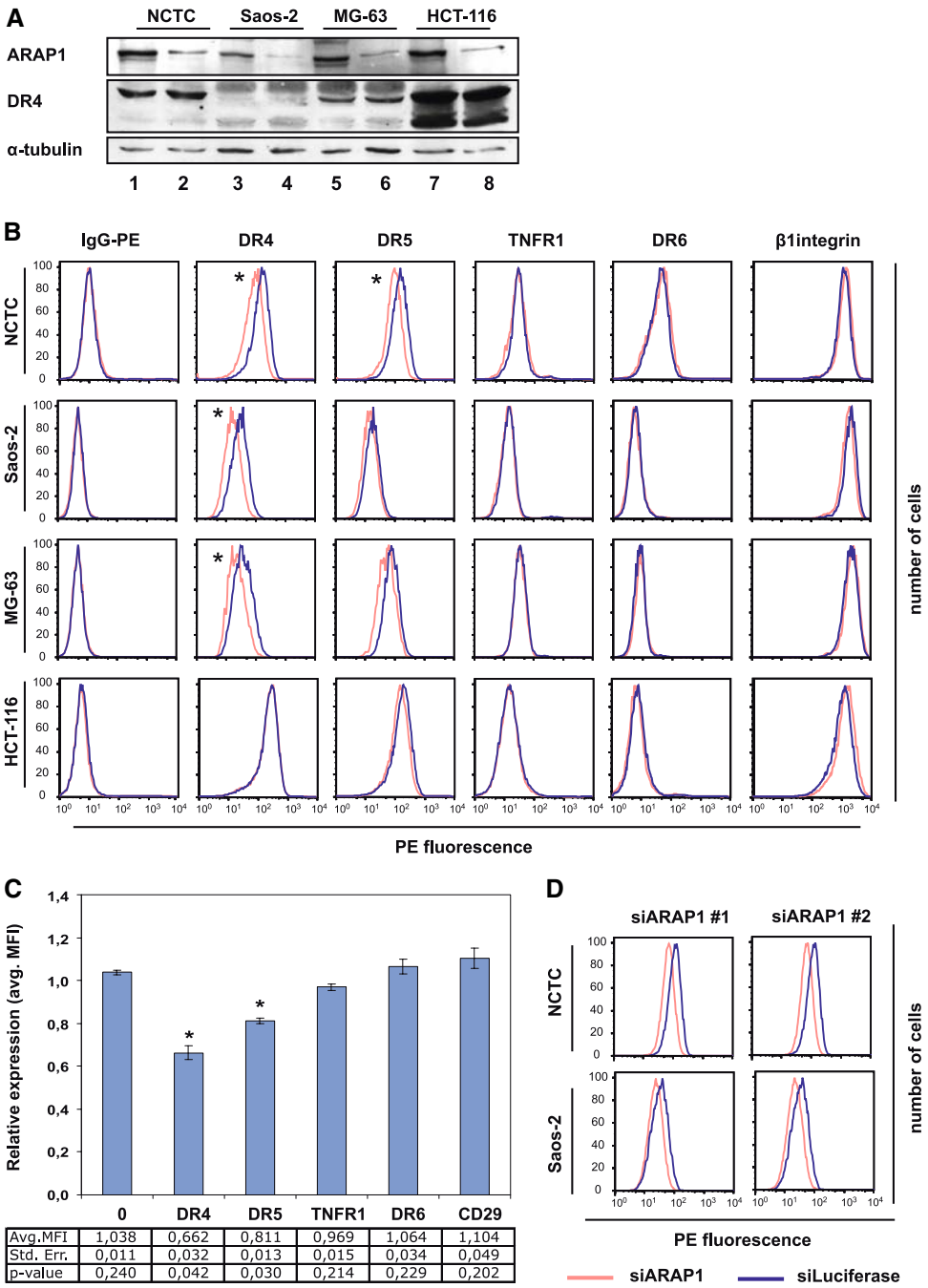

Fig. 5 ARAP1 knockdown compromises the cell surface localization of DR4. (**a**) Cell lysates from NCTC, Saos-2, MG-63 and HCT-116 cell lines transfected either with control luciferase siRNA (lanes 1, 3, 5, 7) or with ARAP1 siGENOME (lanes 2, 4, 6, 8) were stained on Western blots with antibodies against ARAP1, DR4 and α-tubulin (loading control). (**b**) Cells were grown in 24-well plates and transfected either with Luciferase siRNA (blue lines) or ARAP1 siGENOME (red lines). Fifty-two hours after transfection, the cells were harvested and stained with antibodies against DR4, DR5, TNFR1, DR6, β1 integrin or with the secondary GAM-PE antibody only (IgG-PE) and the living cells (Hoechst 33258 negative) were analyzed by flow cytometry. Representative histograms from at least four independent experiments are shown. (**c**) Relative representations of the averaged fluorescence medians of the cell surface expression (MFI siARAP1/MFI siLuc) of selected receptors in NCTC cells from four independent experiments. Standard Errors and statistical significances are shown (* = $P < 0.05$). (**d**) NCTC and Saos-2 cells were transfected with luciferase or two individual ARAP1 siRNAs and analyzed for DR4 cell surface expression as in (**b**)

thus its absence does not have to compromise the binding of phosphatidylinositols to the PH5 domain.

DR4 apparently interacts with ARAP1 through the N-terminal part of its death domain. The death domain of TRAIL receptors is essential for FADD-mediated DISC formation, and it also mediates interaction with RIP [33]. As ARAP1 was not detected at early time points in DR4 immunoprecipitate (Fig. 2c and Suppl. Fig. 2), it seems to be unlikely that it could affect DISC assembly or caspase-8 activation. Though obviously weak and transient, ARAP1 interaction with DR4 and, to a lesser extent, DR5 is likely to be specific for these two death receptors as the apparent interaction motif in the first DR4 α-helix/turn region is conserved only between DR4 and DR5 (Fig. 2b and Suppl. Fig. 2a). This first DR4 α-helix also contains FxDxF motif, which is apparently recognized by α-ear and PHEar domains from clathrin endocytosis accessory proteins AP-2 and Necap, respectively [37, 38]. As this motif overlaps with or is in the proximity of ARAP1-interacting region, it seems to be plausible that ARAP1 could also participate in

regulation of the spontaneous or activated clathrin-dependent endocytosis of DR4. Removal of the C-terminus of DR4 or DR5 (the last 16 or 13 amino acids, respectively) compromised FADD binding to the truncated receptors and suppressed TRAIL-induced apoptosis [39]. This C-terminal truncation apparently affected the structure or orientation of their death domains and also negatively influenced the interaction between DR4 and ARAP1-C (Fig. 2b). In contrast to the published ARAP1 localization in Golgi [28], we detected both transfected and endogenous ARAP1 at the cell membrane where it co-localized with DR4 (Fig. 3a) or co-fractionated in light membrane fractions (Suppl. Fig. 5). DR4 and ARAP1 were also associated within the ER/Golgi compartment, but neither ARAP1 nor DR4 were present in Golgi membrane-containing fractions in a sucrose gradient (Suppl. Fig. 5). Miura et al. published incomplete ARAP1 cDNA lacking the N-terminal SAM domain, and thus this clear discrepancy in ARAP1 cellular localization is likely to be attributed to this missing SAM domain. Moreover, the PH domains of ARAP1 and its relative ARAP3 preferentially interact with plasma membrane bound PtIns(3,4,5)P_3 or PtIns(3,4)P_2 and not with Golgi-associated PtIns phosphates [28, 40]. Thus, it seems likely that transient interaction between DR4 and ARAP1 takes place at the plasma membrane or on the way to the plasma membrane and could affect DR4 localization and/or function.

Transient ARAP1Δexon30 overexpression did not affect either DR4 cell surface expression or TRAIL-induced apoptosis in HEK293FT or HeLa cells. Indeed, analysis of HEL (which express only DR4) clones producing ARAP1Δexon30 confirmed these data (no significant changes in DR4 expression or in the kinetics of TRAIL-induced apoptosis) with one notable exception. Cells with overexpressed ARAP1 significantly more efficiently presented phosphatidyl serine (PS) at their cell surface upon activation of TRAIL-induced apoptosis (Fig. 4). This phenomenon was not accompanied by their increased apoptosis and thus could be related either to ARAP1-mediated enhancement of scramblase or to more efficient suppression of floppase activities. Enhanced PS externalization also occurs independently of apoptosis and could be induced by the aggregation of GPI-anchored proteins or by low levels of tyrosine phosphatase CD45RB in T lymphocytes [41, 42]. ARAP1 overexpression in HeLa cells induced the formation of actin-containing peripheral blebs [28]. The ARAP1 relatives ARAP2/3 bear a high affinity for Arf6 and RhoA and participate in the regulation of focal adhesion and cell spreading [43–45]. Thus, overexpressed ARAP1 can somehow disturb plasma membrane homeostasis and modulate the activities of aminophospholipid-distributing enzymes.

In contrast to cells with overexpressed ARAP1, its siRNA-mediated down-regulation led in several cell lines to the decreased cell surface expression of DR4 as well as to a markedly slower initial phase of TRAIL-induced apoptosis (Figs. 5 and 6). The cell surface localization of non-interacting death receptors (DR6) or other receptors (β1-integrin, HLA-1) was almost or entirely unaffected. Though it was reported that DR4 and other death receptors reside primarily in the trans-Golgi network (TGN) [19], in NCTC cells either overexpressed or endogenous DR4 was predominantly localized in the endoplasmic reticulum and at the plasma membrane (Fig. 3, Suppl. Fig. 5 and not shown). Hence, ARAP1 down-regulation could disturb DR4 trafficking from the intracellular stores to the plasma membrane. We reproducibly observed a correlation between the loss of DR4 plasma membrane localization and its increased accumulation in the ER/Golgi-containing pellet in cells with downregulated ARAP1 (Suppl. Fig. 5). In addition to its Arf1 and RhoA GAP activities, ARAP1 can function as a vesicle coat protein and directly interact with and regulate the trafficking of cargo proteins such as DR4. This was proven for its relative from the AZAP family, ASAP1, which is directly involved together with ARAP3 in the trafficking of the EGF receptor [23, 46]. The other members of this family, AGAP1 and AGAP2, associate with coat-protein complexes AP-3 and AP-1, respectively, and specifically regulate the recycling of the transferrin receptor [47]. Therefore, direct interaction between ARAP1 and DR4 could play a role in establishing and/or stabilizing DR4-containing transport vesicles. Several other reports also point to the specific regulation of DR4 trafficking in normal and transformed cells. A failure in the transport of DR4 to the plasma membrane was behind the acquired resistance of SW480 colon adenocarcinoma cells to TRAIL-induced apoptosis [48]. SiRNA-mediated knockdown of a 72 kDa subunit of the SRP (signal recognition particle) complex suppressed the plasma membrane localization of DR4 but not its total cellular level. No other tested death receptors (DR5, TNFR1 or CD95) were affected by this silencing [49]. Why this trafficking regulation is specific only for DR4 remains to be answered.

The availability of other death receptors such as Fas/CD95 is also regulated at the TGN-plasma membrane trafficking step. p53 activation can transiently increase Fas transport from the Golgi complex to the cytoplasmic membrane [20], and Fas-interacting proteins FAP-1 and dynamin-2 could play an opposite role in this process [50]. In contrast to FAP-1, dynamin-2 enhances Fas transport from the TGN to the cell surface. The regulation of death receptor activities through their mobilization to the cell surface from the TGN appears to be a very effective way to respond rapidly to sudden changes in the environment or within the cell. Bile salts induce rapid Fas-mediated apoptosis of hepatocytes through the enhancement of Fas

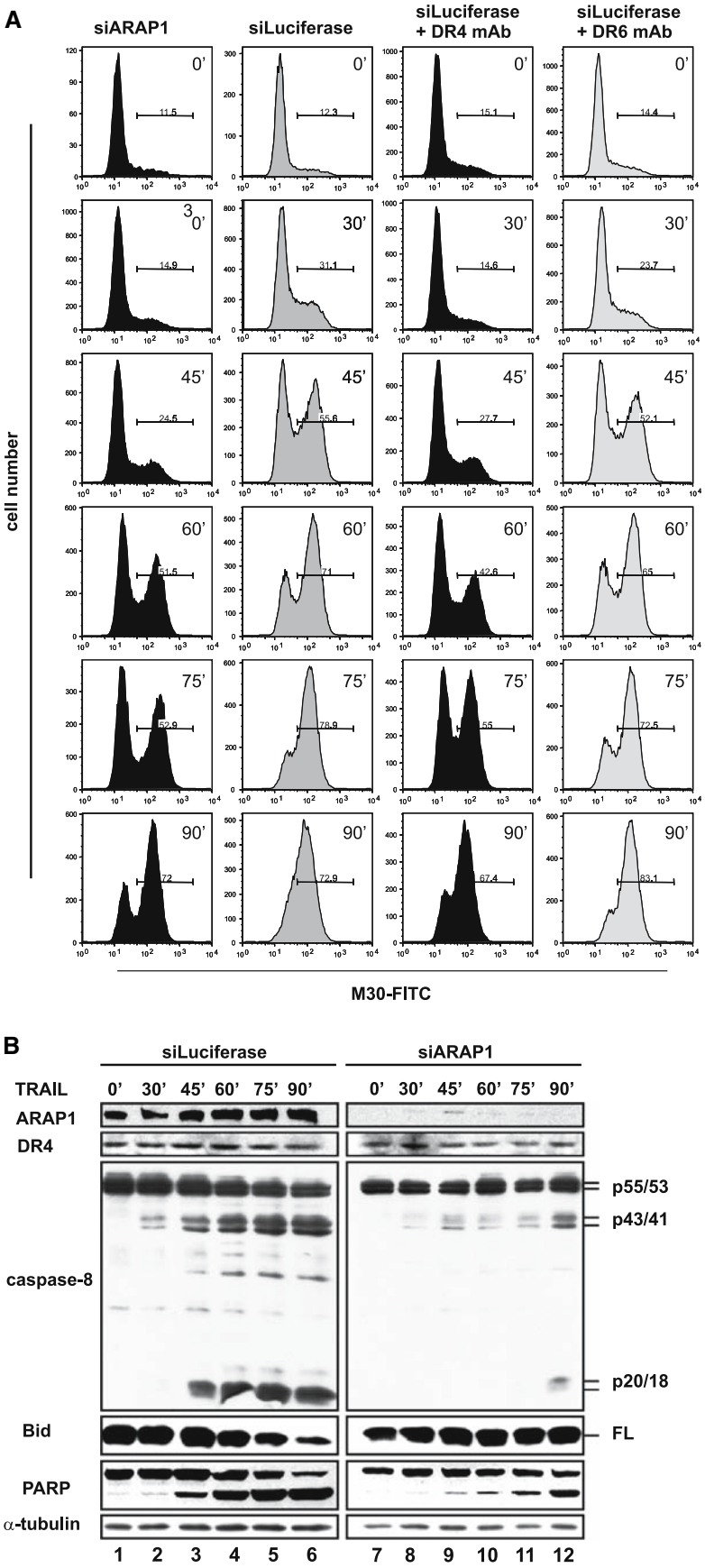

Fig. 6 Downregulation of ARAP1 decelerates TRAIL-induced apoptosis of NCTC cells. NCTC cells were grown in 24-well plates and transfected either with luciferase or with ARAP1 siRNA. Fifty-two hours post-transfection, some of the siLuc transfected cells were pre-incubated with blocking anti-DR4 or anti-DR6 monoclonal antibodies (at a final conc. of 10 μg/ml for 1 h), then the cells were treated with TRAIL (200 ng/ml) for 0, 30, 45, 60, 75, or 90 min, harvested and washed with ice-cold PBS. (**a**) Cells were fixed with ice-cold methanol, stained with FITC-conjugated anti-M30 antibody according to the manufacturer's instructions and analyzed by flow cytometry. The abscissa above the histograms with the indicated percentage represents the percentage of M30-positive, apoptotic cells. (**b**) Total cell lysates of luciferase or ARAP1 siRNA-treated NCTC cells taken at the indicated time points were analyzed by Western blotting using the corresponding antibodies (α-tubulin antibody was used as a loading control)

translocation from the Golgi to the cell membrane [51]. Mobilization of the intracellular stores of death receptors may also represent a self-destruction shortcut during malignant transformation. For example, malignant and normal epithelial cancer cells both express similar levels of DR4, but only transformed PC3 or JCA-1 cells have DR4 present at the cell surface and are sensitive to TRAIL-induced apoptosis [52].

In addition to possible ARAP1-mediated enhancement of DR4 transport to the plasma membrane, its co-localization with DR4 at this membrane suggests that ARAP1 could also affect DR4 plasma membrane stability or its correct localization. ARAP1 could be an important mediator of Arf or Rho GTPase-regulated trafficking of proapoptotic TRAIL receptors from the Golgi/trans-Golgi to the membrane and/or their endocytosis. The precise mechanism underlying ARAP1-affected DR4/DR5 trafficking is currently under investigation.

Conclusions

The Rho and Arf GAP adapter protein ARAP1 specifically interacts with the membrane proximal part of the death domain of DR4/TRAIL-R1 in both yeast and human cells. In contrast to the originally published sequence, DR4-interacting ARAP1 variant ARAP1Δexon30 lacks the alternatively spliced exon 30. Transfected ARAP1 co-precipitates with DR4 and co-localizes with it at the ER/Golgi and at the plasma membrane. Upon TRAIL treatment, internalized DR4 is endocytosed and subsequently co-localizes with ARAP1 in early endosomes. Endogenous DR4 and partially processed ARAP1 also co-immunoprecipitate from the lysate of TRAIL-treated NCTC cells. siRNA-mediated downregulation of ARAP1 markedly and specifically reduces the cell-surface localization of DR4 (and partly of DR5) in most of the tested human cell lines. The downregulation of the surface expression of DR4 results in slower TRAIL-induced apoptosis in two tested cell lines, comparable to the rate obtained by blocking the DR4 receptor with a monoclonal antibody. The stable overexpression of ARAP1 in HEL cells does not change the surface expression of DR4, but leads to more rapid exposure of phosphatidyl serine on the cell surface during TRAIL-induced apoptosis. ARAP1 could be an important regulator of DR4 trafficking from the ER/Golgi to the cellular membrane.

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