Functional Characterization of the CFTR R Domain Using CFTR/MDR1 Hybrid and Deletion Constructs†

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Received July 1, 1999; Revised Manuscript Received September 2, 1999

ABSTRACT: To improve our insight into the structure and function of the CFTR R domain, deletion and hybrid constructs in which different parts of the R domain were deleted or replaced by the MDR1 linker domain, and vice versa, were made. Replacement of the linker domain by the R domain did not result in a decrease and replacement of the CFTR R domain by the linker domain did not result in an increase of maturation efficiency, when compared to the respective wild-type proteins. This indicates that the R domain is not responsible for the high degree of degradation observed for CFTR translation products in the ER, but rather the overall structure or sequences located outside the R domain. Replacing the C-terminal part of the R domain (amino acids 780−830) by the MDR1 linker domain resulted in the appearance of PKA-dependent whole cell chloride currents which were not significantly different from wild-type CFTR currents. This might indicate that the PKA sites present in the linker domain are functional and that not the exact sequence of the C-terminal part of the R domain is important, but rather the presence of PKA sites and the length. Moreover, when this hybrid construct was PKC-stimulated, chloride currents were activated. Although these PKC-induced currents were lower than the PKA-induced ones, this again indicates that the linker domain is functional in this hybrid construct. Taken together, these results suggest that the MDR1 linker domain can substitute for part of the regulatory domain of the CFTR protein.

The ATP binding cassette (ABC)[1] transporter family contains multiple transporter molecules, found in prokaryotes and eukaryotes. Two of these family members found in humans have a very similar structure: the cystic fibrosis transmembrane conductance regulator (CFTR) and P-glycoprotein (MDR1) (Figure 1). Both proteins contain a repeat of one transmembrane domain (TMD), composed of six transmembrane helices (TM), coupled to one nucleotide binding domain (NBD) (*1*−*3*). These repeats are separated by different domains in both proteins: an R domain of 332 amino acids containing multiple PKA and PKC consensus sites in CFTR (*4*), and a linker (L) domain of 46 amino acids containing multiple PKA, PKC, and P-glycoprotein specific V1-kinase consensus sites in MDR1 (Figure 1) (*5*).

The importance of phosphorylation for function seems to be different for both proteins. CFTR mainly functions as a chloride channel (*6*−*8*) and as a regulator of other ion channels (*9*−*12*). Phosphorylation by protein kinase A is important for both functions. Opening of the chloride channel requires phosphorylation of the R domain by PKA and subsequent binding and hydrolysis of ATP by both nucleotide binding domains (*13*). The R domain contains 10 PKA sites, and the degree of phosphorylation determines the affinity of both nucleotide binding domains for ATP, resulting in fine-tuning of the activity of the chloride channel through phosphorylation of a specific combination of PKA sites present in the R domain (*14*, *15*). The precise working mechanism and the R domain sequences necessary to confer these complex regulatory properties remain, however, unknown. Phosphorylation by PKC alone is not enough to activate the chloride channel but enhances the effect of PKA activation, although contradictory data exist concerning this topic (*16*, *17*). Regulation of ORCC (*18*) and ATP channels (*12*) requires a CFTR protein with an intact R domain, indicating that phosphorylation of the R domain is also important for these functions.

† These investigations have been supported by the Alphonse and Jean Forton grant of the "Koning Boudewijn Stichting", by grant G.0237.95 from the "Fonds voor Wetenschappelijk Onderzoek" (to B.N.), by C.O.F./96/22-A0659 (to B.N.), by G.O.A. 99/07, by IUAP Nr. 3P4/23, and by the European Commission (BMH4-CT96-0602). A.V. is a fellow of "Het Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch onderzoek in de industrie".

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[1] Abbreviations: aa, amino acid(s); ABC transporter, ATP binding cassette transporter; cAMP, adenosine 3′,5′-cyclic monophosphate; CFTR, cystic fibrosis transmembrane conductance regulator; COS1, SV40-transformed African green monkey kidney cell line; DMEM/F12, Dulbecco's modified Eagle's medium/Nutrient mix F-12; dNTP's, 2′-deoxynucleotide 5′-triphosphates; ENaC, amiloride-sensitive epithelial sodium channel; ER, endoplasmic reticulum; GFP, green fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; MDR1, P-glycoprotein; NBD, nucleotide binding domain; ORCC, outwardly rectifying chloride channel; PCR, polymerase chain reaction; PDBu, 4β-phorbol 12,13-dibutyrate; PKA, protein kinase A; PKC, protein kinase C; R domain, regulatory domain; RPMI 1640, Roswell Park Memorial Institute medium type 1640; TBE, Tris−borate/EDTA electrophoresis buffer; TM, transmembrane helix; TMD, transmembrane domain; VRAC, volume-regulated anion channel.

10.1021/bi991520d CCC: $18.00
Published on Web 10/20/1999

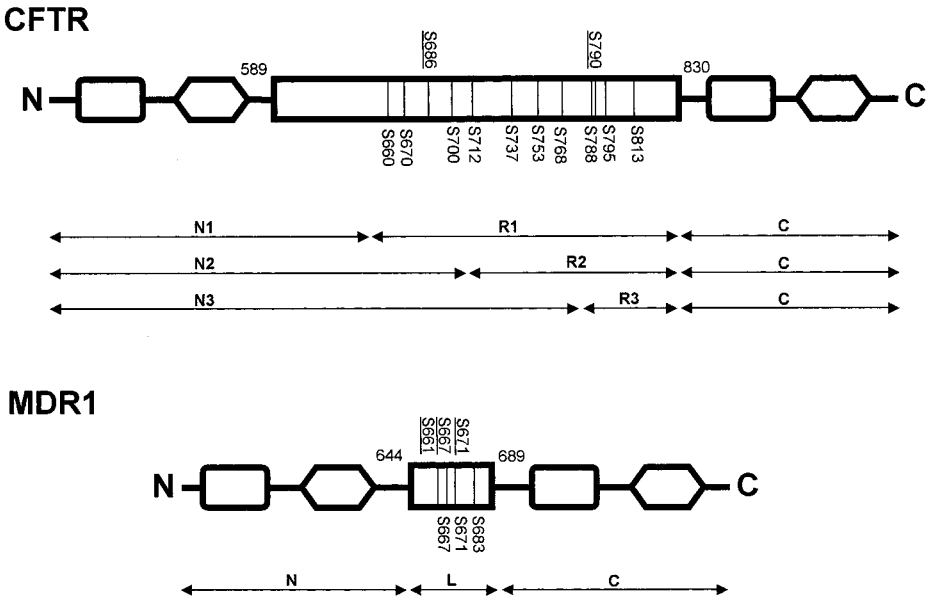

FIGURE 1: Schematic representation of the CFTR and MDR1 proteins. Both proteins are represented as linear molecules with the transmembrane domains shown as rounded rectangles, the nucleotide binding domains as diamonds, and the R domain of CFTR and the linker domain of MDR1 as rectangles. The first and last amino acids of the R domain [according to Zielenski et al. (*41*)] and the linker domain [according to Chen et al. (*1*)] are indicated. The PKA sites and PKC sites (underlined) present in the R domain and linker domain are written respectively under and above the figures. The PKC consensus sites present in the MDR1 linker domain are also recognized by the P-glycoprotein-specific V1-kinase. The fragments used for the construction of the different hybrid and deletion constructs are drawn for both proteins and named as in Figure 2.

P-glycoprotein mainly functions as a transporter of hydrophobic compounds, giving rise to multidrug resistance phenotypes in cancer cells (*19*). To confer this multidrug resistance phenotype, MDR1 extrudes toxic drugs, which entered the cytosol, out of the cell using energy produced through ATP hydrolysis at its nucleotide binding domains. In vivo phosphorylation of the linker domain has been detected at PKC sites, but phosphorylation seems not to be important for export of hydrophobic compounds (*20*), leaving the question about its function open.

During translation, both proteins enter the endoplasmic reticulum where they are core-glycosylated. With the aid of chaperones such as Hsp70 and calnexin, both proteins fold into a mature form that moves through the Golgi-stacks, where the carbohydrate groups further mature, to the cell membrane (*21*−*23*). The folding process in the ER is inefficient since only 25% of the CFTR translation products are able to leave the ER for transport to the cell membrane (*24*). The remaining 75% is degraded in a ubiquitin-dependent way by the 26S proteasome (*25*). The reason for this inefficient folding process is unknown. The maturation of MDR1 is much more efficient, since almost no degradation of the core-glycosylated form is observed (*26*). The main difference between both proteins is located in the cytosolic domain, which links the first nucleotide binding domain to the second transmembrane domain. Indeed, CFTR carries a bulky R domain, whereas MDR1 has only a short linker domain at this site.

It is clear that the role of the R domain in CFTR channel regulation and gating, and maturation is still obscure. To investigate the potential importance of the R domain in the significant degradation of wild-type CFTR translation products in the ER, hybrid and deletion constructs of different length containing sequences of *CFTR* and *MDR1* were produced, and their maturation patterns were determined. In addition, these constructs were used to analyze the potential of the MDR1 linker domain to function as, and to substitute for, the "regulatory domain" in CFTR channel gating.

MATERIALS AND METHODS

1. Construction of CFTR−*MDR1 Hybrids.* Hybrid *CFTR*−*MDR1* constructs were generated using a PCR-based technique. Each template, the *CFTR* (kindly provided by Transgene S.A.) and *MDR1* coding regions (kindly provided by P. Sonneveld; *27*) inserted in the pcDNA3 vector (*28*), was amplified by PCR in three fragments: an N-terminal part, a central part containing the linker domain of *MDR1* or part of the R domain of *CFTR*, and the C-terminal part. The primers (see Table 1) used for amplification contained adaptor sequences harboring recognition sites for *Pst*I such that, after digestion, blunt-ending, and ligation, in-frame constructs were obtained. To facilitate subsequent subcloning steps, the sense primers of the N-terminal parts contained a *Nhe*I site, and the antisense primers of the C-terminal parts contained *Xho*I (of *CFTR*) or *Sal*I (of *MDR1*) sites. The different fragments were amplified with Pwo DNA Polymerase (Boehringer Mannheim), a heat-stable DNA polymerase with proofreading activity, in a hot start PCR protocol. To this, a bottom solution (50 μL) containing 20 mM Tris-HCl, pH 8.85, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 30 pmol of left and right primer, and 10 ng of template DNA was separated from the top solution (50 μL), containing 0.4 mM dNTP's and 5 units of Pwo DNA Polymerase, by a Dyna Wax F-600 seal. The following temperature profile was used: 2 min at 95 °C; 30 cycles of 30 s at 95 °C, 40 s at 55 °C, and 1 min 30 s at 72 °C; 6 min at 72 °C; 15 min at 4 °C, in a GENE Amp PCR system 2400 (Perkin-Elmer). The PCR fragments were purified on and isolated from a 1% TBE−agarose gel using the Qiaquick Gel Extraction Kit (Qiagen). They were then blunt-ended

Table 1: Summary of the Primers Used for Amplification of the Different Fragments and the Amino Acid Changes Introduced Due to the Insertion of *Pst*I Sites[a]

NAME	AMPLIFIED FRAGMENTS (A.A)	PRIMERS		INTRODUCED AMINO ACID
CFTR-N1	*Nhe*I-5' - 650-*Pst*I	P.pcDNA3-867.5 (*Nhe*I):	5'- GAA GCT AGC ACG ACT CAC TAT AGG GAG ACC -3'	none
		P.CFTR-2082.3 (*Pst*I):	5'- AAT CTG CAG AAA GAA TCA CAT CCC ATG AG -3'	
CFTR-N2	*Nhe*I-5' - 707-*Pst*I	P.pcDNA3-867.5 (*Nhe*I):	5'- GAA GCT AGC ACG ACT CAC TAT AGG GAG ACC -3'	none
		P.CFTR-2252.3 (*Pst*I):	5'- TTT CTG CAG GAG TTG ATT GGA TTG AGA ATA G -3'	
CFTR-N3	*Nhe*I-5' -779-*Pst*I	P.pcDNA3-867.5 (*Nhe*I):	5'- GAA GCT AGC ACG ACT CAC TAT AGG GAG ACC -3'	Q779H
		P.CFTR-2468.3 (*Pst*I):	5'- TTC CTG CAG TGG TTA ACT GAG TGT GTC A -3'	
CFTR-R1	*Pst*I-651 - 830-*Pst*I	P.CFTR-2083.5 (*Pst*I):	5'- ATT CTG CAG ACC AAT TTA GTG CAG AAA GA -3'	K830N
		P.CFTR-2622.3 (*Pst*I):	5'- AAG CTG CAG TTT AAG TCT TCT TCG TTA ATT TC-3'	
CFTR-R2	*Pst*I-708 - 830-*Pst*I	P.CFTR-2255.5 (*Pst*I):	5'- TCA CTG CAG TAC GAA AAT TTT CCA TTG TGC -3'	I708V/K830N
		P.CFTR-2622.3 (*Pst*I):	5'- AAG CTG CAG TTT AAG TCT TCT TCG TTA ATT TC-3'	
CFTR-R3	*Pst*I-780 - 830-*Pst*I	P.CFTR-2469.5 (*Pst*I):	5'- TAC TGC AGG TCA GAA CAT TCA CCG AAA G -3'	K830N
		P.CFTR-2622.3 (*Pst*I):	5'- AAG CTG CAG TTT AAG TCT TCT TCG TTA ATT TC-3'	
CFTR-C	*Pst*I-831 - 3'-*Xho*I	P.CFTR-2623.5 (*Pst*I):	5'- ACT CTG CAG AGT GCT TTT TTG ATG ATA TGG -3'	none
		P.pcDNA3-995.3 (*Nsi*I):	5'- CTA ATG CAT AGG GCC CTC TAG ATG CAT -3'	
MDR1-N	*Nhe*I-5' - 643-*Pst*I	P.pcDNA3-867.5 (*Nhe*I):	5'- GAA GCT AGC ACG ACT CAC TAT AGG GAG ACC -3'	E643D
		P.MDR1-2353.3 (*Pst*I):	5'- CTT CTG CAG TCA TCA GCT GCA TTT TCT AAT TC-3'	
MDR1-L	*Pst*I-644 - 689-*Pst*I	P.MDR1-2354.5 (*Pst*I):	5'- CTG CTG CAG CCA AAA GTG AAA TTG ATG CC -3'	S644A
		P.MDR1-2491.3 (*Pst*I):	5'- ATA CTG CAG TCC AGA GCC TCT TTG GTA CT -3'	
MDR1-C	*Pst*I-690 - 3'-*Sal*I	P.MDR1-2492.5 (*Pst*I):	5'- CTC CTG CAG AAA GTA TAC CTC CAG TTT CC -3'	none
		P.pcDNA3-987.3 (*Nsi*I):	5'- TAG ATG CAT CTA GAT GCA TGG TCG AC -3'	

[a] In the second column, the amino acid boundaries of the amplified coding sequences are noted between the restriction sites that were introduced at the 5 and 3 prime ends of the fragments. 5′ indicates that the fragments start 5 prime from the coding sequences; 3′ that they end 3 prime from the coding sequences. Amino acids were numbered according to Zielenski et al. (*41*) for CFTR and according to Chen et al. (*1*) for MDR1. The primers are named according to the 5 prime nucleotide that matches with the template; consensus sites for restriction enzymes present in the primers are underlined. The extension .5 indicates that the primer amplifies the sense strand of the fragment; the extension .3 indicates amplification of the antisense strand.

with T_4 DNA polymerase (Pharmacia Biotech), phosphorylated with T_4 polynucleotide kinase (Pharmacia Biotech), and ligated (T_4 DNA ligase; New England Biolabs) in the prokaryotic vector pUC18/*Sma*I/BAP (Pharmacia Biotech), according to the protocol of the manufacturer. Between the different enzymatic treatments, the modified DNA was purified using the Qiaquick PCR Purification Kit (Qiagen). The obtained ligation mixture was electroporated in $\pm 1.2 \times 10^9$ *XL1 Blue E. coli* cells that were plated, together with 1 μL of 1 M isopropylthio-β-D-galactoside (Boehringer Mannheim) and 50 μL of 0.05 M 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Gibco BRL Life Technologies), on TYB−agar plates containing 100 μg/mL ampicillin. To check the integrity of the cloned PCR products, the different inserts were sequenced using dideoxy-sequencing techniques (AutoRead Sequencing Kit, Pharmacia). Different approaches were used for the production of *CFTR*−linker constructs and *MDR1*−R domain constructs.

To make *CFTR* constructs, the linker of *MDR1* was isolated from pUC18 constructs using *Pst*I (Boehringer Mannheim) digestion and ligated (T_4 DNA ligase, New England Biolabs) into *Pst*I-linearized (Boehringer Mannheim) and, with bovine alkaline phosphatase (Boehringer Mannheim) dephosphorylated, pUC18 vectors containing the N-terminal fragment of *CFTR*. To remove a *Pst*I site present at the boundary between the N-terminal part and the linker, the generated constructs were partially digested with *Pst*I, blunt-ended with T_4 DNA polymerase (Pharmacia Biotech), and ligated with T_4 DNA ligase (New England Biolabs). For insertion of the C-terminal part into the generated constructs,

the constructs were linearized with *Pst*I (Boehringer Mannheim), blunt-ended with T_4 DNA polymerase (Pharmacia Biotech), and dephosphorylated (bovine alkaline phosphatase; Boehringer Mannheim). The C-terminal part was isolated from pUC18 using *Pst*I (Boehringer Mannheim) and *Nsi*I (Gibco BRL Life Technologies), blunt-ended (T_4 DNA polymerase; Pharmacia Biotech), and ligated in the linear vector using T_4 DNA ligase (New England Biolabs). When constructs containing only the N-terminal and C-terminal parts of *CFTR* were needed, the cloned C-terminal part was isolated from pUC18 using *Pst*I (Boehringer Mannheim) and *Nsi*I (Gibco BRL Life Technologies) digestions, blunt-ended (T_4 DNA polymerase; Pharmacia Biotech), and subsequently ligated with T_4 DNA ligase (New England Biolabs) into *Pst*I-linearized (Boehringer Mannheim), blunt-ended (T_4 DNA polymerase; Pharmacia Biotech), and dephosphorylated (bovine alkaline phosphatase; Boehringer Mannheim) pUC18 constructs containing the N-terminal part.

To make *MDR1* constructs, the C-terminal part of *MDR1* was inserted into *Pst*I (Boehringer Mannheim) digested and dephosphorylated (bovine alkaline phosphatase; Boehringer Mannheim) pUC18 vectors containing the N-terminal *MDR1* part. Since the C-terminal part of *MDR1* contained an internal *Pst*I site, a complete digestion with *Nsi*I (Gibco BRL) and a partial digestion with *Pst*I were performed to isolate the intact fragment. To insert the R domain, the R domain of *CFTR* was isolated from pUC18 constructs using *Pst*I digestion (Boehringer Mannheim) and blunt-ended (T_4 DNA polymerase; Pharmacia Biotech). The *MDR1* construct containing the N- and the C-terminal parts, separated by a *Pst*I site, was linearized using a partial *Pst*I digestion. This partial digestion was needed since the C-terminal part of *MDR1* contained an internal *Pst*I site. The obtained linearized fragment was blunt-ended (T_4 DNA polymerase; Pharmacia Biotech) and dephosphorylated with bovine alkaline phosphatase (Boehringer Mannheim). The isolated R domain was subsequently inserted (T_4 DNA ligase; New England Biolabs) into the linearized pUC18 *MDR1* construct. To obtain a construct containing only the N- and C-terminal fragments of *MDR1*, the partially digested and blunt-ended vector was close-ligated. All recombinant constructs obtained contained only minor amino acid changes at the ligation boundaries: no changes of polarity were introduced (Table 1).

To express *MDR1/CFTR* deletion and hybrid constructs in eukaryotic cells, the *MDR1* inserts were isolated from pUC18 as *Nhe*I (Boehringer Mannheim)−*Sal*I (Gibco BRL Life Technologies) fragments, and the *CFTR* inserts as *Nhe*I (Boehringer Mannheim)−*Xho*I (Boehringer Mannheim) fragments, and inserted into *Nhe*I (Boehringer Mannheim)−*Xho*I (Boehringer Mannheim) linearized pcGFP-IRES-LT1 (kindly provided by J. Eggermont; *29*). The inserts of the GFP vector were completely sequenced in order to rule out any mutagenesis event that might have occurred during the different cloning steps.

2. Expression, Pulse−Chase, and Immunoprecipitation of CFTR. Twenty micrograms of plasmid DNA was electroporated (Biorad Gene Pulser, Biorad Laboratories) in $(1.5-3) \times 10^7$ COS1 cells. The transfected cells were cultured at 37 °C in DMEM F12 (Gibco BRL Life Technologies) supplemented with 10% fetal bovine serum (HyClone Laboratories) and 72 h later used in pulse−chase experiments, according to the protocol described in (*28*). Briefly, cells were starved for 30 min in RPMI 1640 medium (Gibco BRL Life Technologies) without methionine and cysteine, labeled during a 30 min pulse in RPMI 1640 supplemented with 100 μCi/mL [^{35}S]methionine and [^{35}S]cysteine (ICN Pharmaceuticals), and finally chased for different time periods in DMEM F12 supplemented with 10% fetal bovine serum. Cells were scraped in 10 mL of Tris−NaCl (20 mM Tris, 150 mM NaCl, pH 7.4) supplemented with protease inhibitors (10 μg/mL soybean trypsin inhibitor, 0.5 μg/mL leupeptin, 0.5 μg/mL antipain, 0.5 μg/mL pepstatin, 0.5 μg/mL chymostatin, and 0.5 mM phenylmethylsulfonyl fluoride). The cells were isolated by centrifugation (10 min at 800*g*) and subsequently lysed by sonication in 800 μL of ice-cold IPPA buffer (20 mM Tris, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, pH 7.4) supplemented with protease inhibitors (20 μg/mL soybean trypsin inhibitor, 1 μg/mL leupeptin, 1 μg/mL antipain, 1 μg/mL pepstatin, 1 μg/mL chymostatin, and 1 mM phenylmethylsulfonyl fluoride). CFTR proteins, present in the supernatant, were affinity-purified by incubation overnight with 1 μg of anti-CFTR monoclonal antibody directed against the C-terminal part of CFTR (Genzyme Diagnostics) or MDR1 proteins with 1.5 μg of anti-MDR1 monoclonal antibody directed against the C-terminal part of MDR1 (C219; DAKO) on a rotor at 4 °C. The immunocomplexes were isolated with 40 μL of protein A−Sepharose CL-4B beads. After addition of 50 μL of loading buffer (1% β-mercaptoethanol, 16 mM Tris-HCl, pH 6.8, 4% SDS, bromophenol blue, and 10% glycerol), the purified protein complexes were incubated at room temperature for 15 min and subsequently loaded on a 4−12% SDS gel (Novex). After being dried, the gel was exposed at −70 °C to a light-sensitive film for 3−4 h.

3. Whole Cell Recordings. A total of $(1.5-3) \times 10^7$ COS1 cells were transfected with 20 μg of the different pcINeo/GFP−CFTR constructs and, 30 h later, seeded on 18 mm glass coverslips at a density of 5000 cells per coverslip in DMEM F12 supplemented with 10% fetal bovine serum, using standard cell culture techniques. Forty-eight to seventy-two hours after transfection, the coverslips were placed in a recording chamber mounted on the stage of an Axiovert 100 microscope (Zeiss, Oberkochen, Germany) containing a Xenon light source and epifluorescence optics (Zeiss, XBO 75 and Zeiss-EPI unit fluorescence condenser). A band-pass filter (Zeiss BP 450−490) was used for excitation of GFP proteins. Excitation was done via a dichroic mirror (Zeiss FT 510). Light emitted by GFP-expressing cells passed through a 520 nm long-pass filter (Zeiss LP 520) and was visually detected. Only cells emitting green fluorescent light after excitation, and consequently expressing GFP and CFTR proteins, were used for measurements. Rapid exchange and extracellular application of drugs occurred via a multibarreled pipet connected to solution reservoirs, and was controlled by a set of magnetic valves. Patch electrodes with a resistance between 2 and 5 MΩ were pulled from capillary tubes (Science Products GMBH, Hofheim, Germany) on a DMZ-universal puller (Zeitz-Instruments, Ausburg, Germany). An Ag−AgCl wire was used as reference electrode. Membrane currents were recorded using an EPC-7 patch-clamp amplifier (List Electronic, Lambrecht/Pfalz, Germany) and filtered with an eight-pole Bessel filter (Kemo, Bekenham Kent, U.K.). For control of voltage-clamp protocols and data acquisition, the pCLAMP 6 software (Axon Instruments, Foster City,

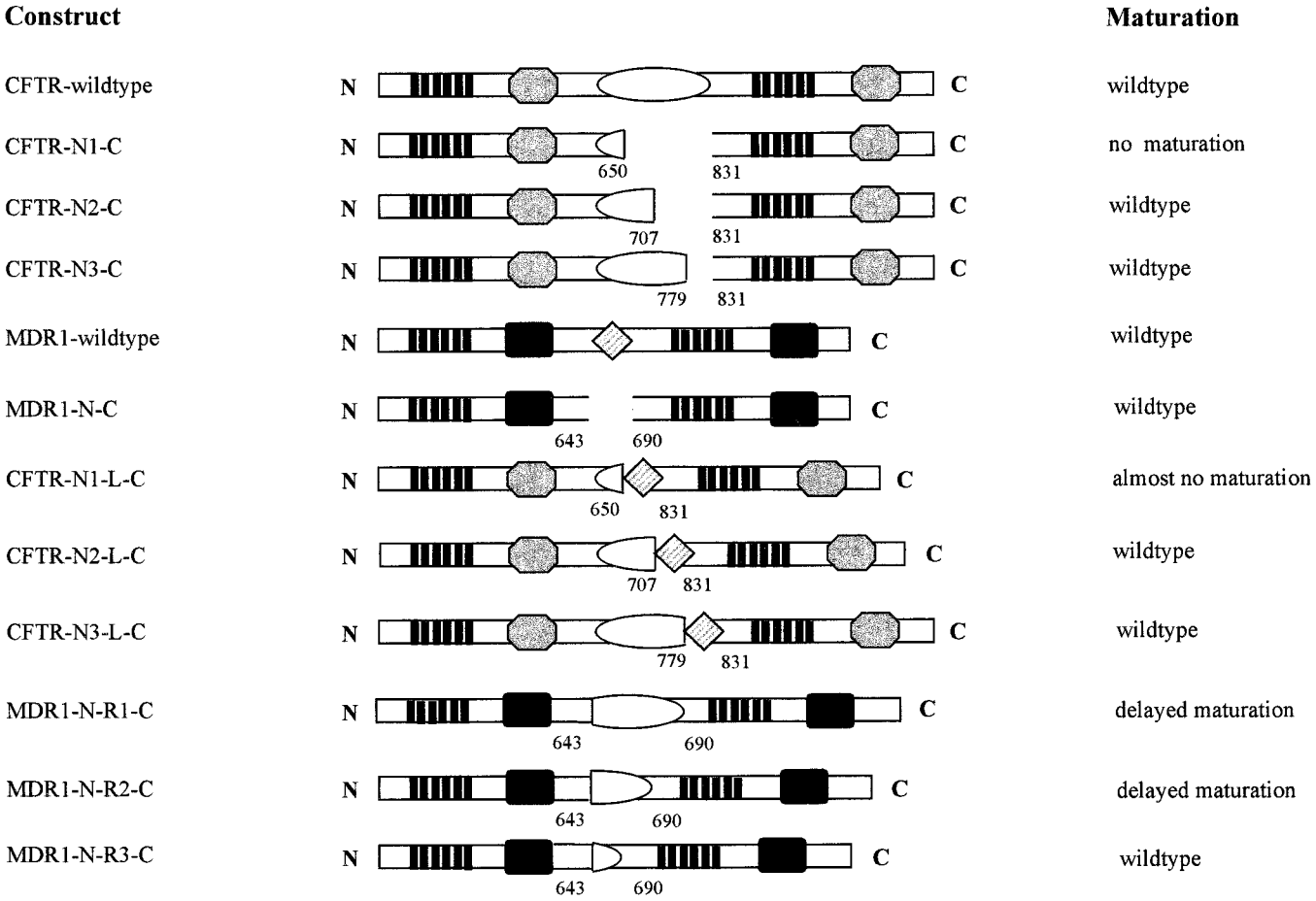

FIGURE 2: Synopsis of the structures of and the maturation patterns obtained for the different deletion and hybrid constructs. The left column gives the names of the constructs that are schematically represented in the middle column. The first part of the name represents the backbone sequence, containing the N-terminal (N) and the C-terminal parts (C) of *CFTR* or *MDR1*, in which deletions and/or insertions were made. L indicates insertion of the linker domain of *MDR1*, and R indicates insertion of the R domain of *CFTR*. As can be seen in the figure, three different parts were used as N-terminal parts and R domains of *CFTR*. They have been named N1 (aa 1−650), N2 (aa 1−707), and N3 (aa 1−779) and R1 (aa 651−830), R2 (aa 708−830), and R3 (aa 780−830). In the schematic representation, the positions of the 3′ end of the N-terminal parts and the 5′ end of the C-terminal parts are given. A comparison of the maturation pattern obtained for the different constructs with the maturation pattern obtained for the wild-type "backbone" construct is given in the last column. The transmembrane helices of CFTR and MDR1 are represented by black bars, the nucleotide binding domains of CFTR by octagons and of MDR1 by rectangles, the R domain of CFTR by an ellipse, and the linker domain of MDR1 by a diamond. Deleted parts are represented by empty spaces in the construct.

CA) run on an IBM-compatible PC, which was connected to the amplifier via a TL-1 DMA interface (Axon Instruments, Foster City, CA), was used. All electrophysiological measurements were done at room temperature in the whole cell mode of the patch-clamp technique. The cell capacitance and series resistance were assessed using the analogue compensation circuit of the EPC amplifier. Between 50 and 80% of the series resistance was electronically compensated. A ramp protocol, consisting of one step to −100 mV and a 400 mS linear voltage ramp to +100 mV, was applied every 10 s from a holding potential of −20 mV. Current−voltage ($I-V$) relations were obtained from the currents measured during the linear voltage ramp. Currents were sampled at 0.5 ms intervals and filtered at 5 kHz. The standard bath solution contained (in mM): 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, titrated with NaOH to pH 7.4. KCl was substituted with 5 mM CsCl 5 min before the experimental protocol was started. The standard pipet solution contained (in mM): 20 CsCl, 20 tetraethylammonium chloride, 100 cesium aspartate, 4 Mg-ATP, 4 EGTA, 5 HEPES, titrated to pH 7.2 with CsOH. The channels were activated through addition of 100 μM IBMX and 10 μM forskolin or 1 μM PDBu to the bath (external) solution. All data were analyzed by Student's t-test. A value of $P < 0.05$ was considered as significant. Data are reported as mean ± SEM.

RESULTS

To determine the role of the R domain in the "normal" degradation of CFTR translation products in the endoplasmic reticulum, the linker domain of MDR1 and the R domain of CFTR were deleted from their respective constructs, or swapped (Figure 2). The linker domain was defined by amino acids 644−689 of MDR1 (*5*). Recent structural models, physicochemical evidence, and protein maturation studies have indicated that the limits of the R domain may be different from what has been accepted thus far (*30*, *31*). Therefore, different parts of exon 13 of CFTR were deleted. First, amino acids 651−830 (R1) were deleted. Second, an R domain deletion construct of amino acids 708 up to 830 (R2) was studied. The influence of this deletion on CFTR anion transport activity has previously been studied by Rich et al. (*32*). The deleted part contains multiple phosphorylation sites for protein kinase A and has no homology with MDR1.

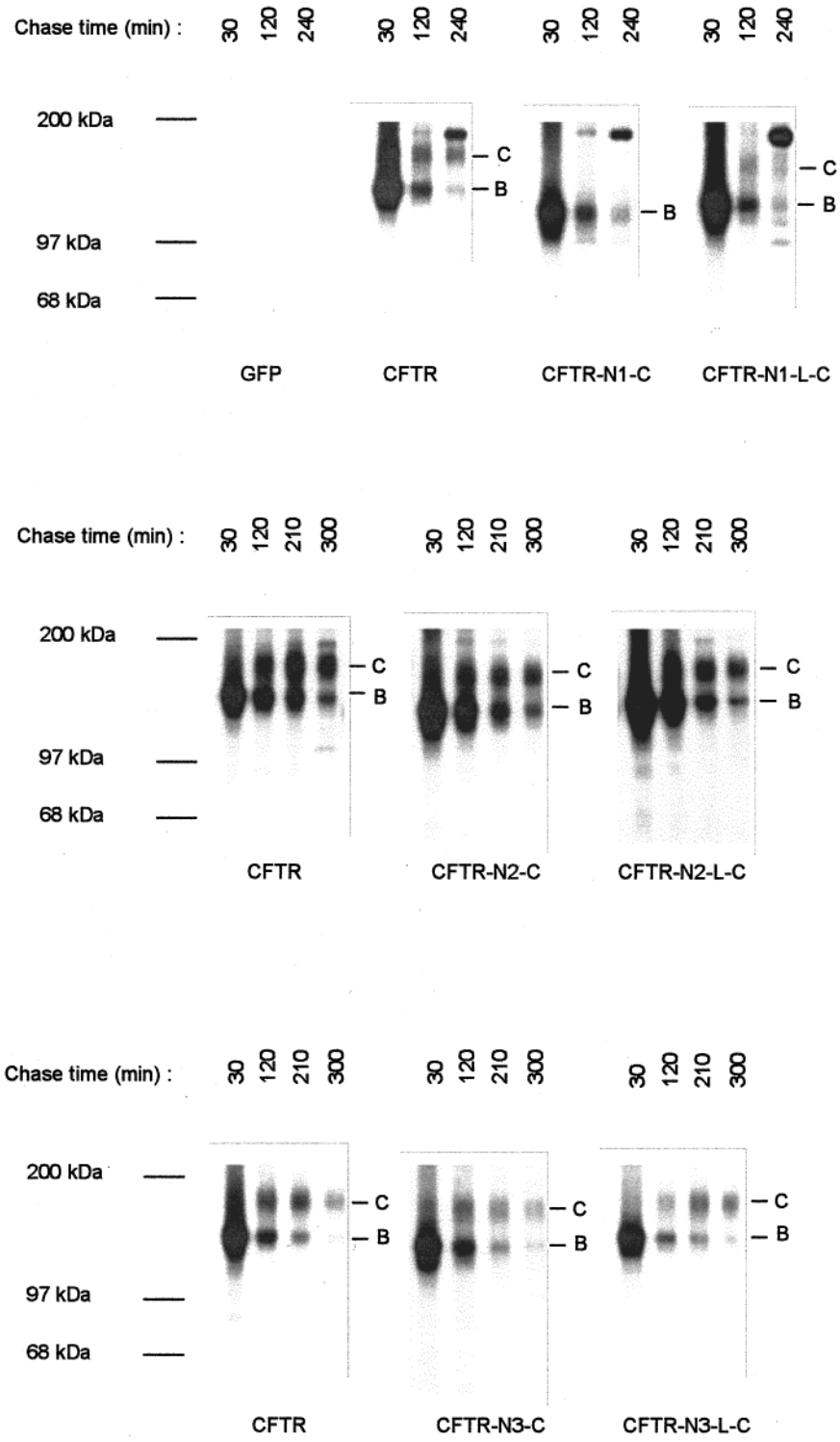

FIGURE 3: Pulse−chase and immunoprecipitation of CFTR proteins from COS1 cells transiently expressing wild-type, deletion, and hybrid CFTR proteins. Proteins were labeled with [^{35}S]methionine and [^{35}S]cysteine and chased for the indicated time periods, and CFTR proteins were immunoprecipitated with an antibody directed against the C-terminal part of CFTR. The immunopurified proteins were separated on a 4−12% SDS gel and visualized by autoradiography. The core-glycosylated B-forms and the mature C-forms are indicated on the right for each construct. The names of the different expressed constructs are given (nomenclature of Figure 2); GFP indicates empty vector. The experiment was repeated with the same result.

Third, a short fragment of the R domain, encompassing amino acids 780−830 (R3), was deleted or substituted. This fragment has the same length as the MDR1 linker domain.

1. Maturation of CFTR Deletion and Hybrid Constructs. When wild-type CFTR was expressed in COS1 cells, only a small fraction of the translation products was converted into the mature C form (Figure 3). When amino acids 650−830 (R1) were deleted (CFTR-N1-C), maturation was completely blocked since no mature proteins could be detected. Replacement of this fragment by the linker domain of MDR1 (CFTR-N1-L-C) could only slightly reverse this maturation block; the amount of protein that appeared as mature C-band was much lower than what was observed for wild-type CFTR. When a smaller part of CFTR [aa 708−830 (R2) or

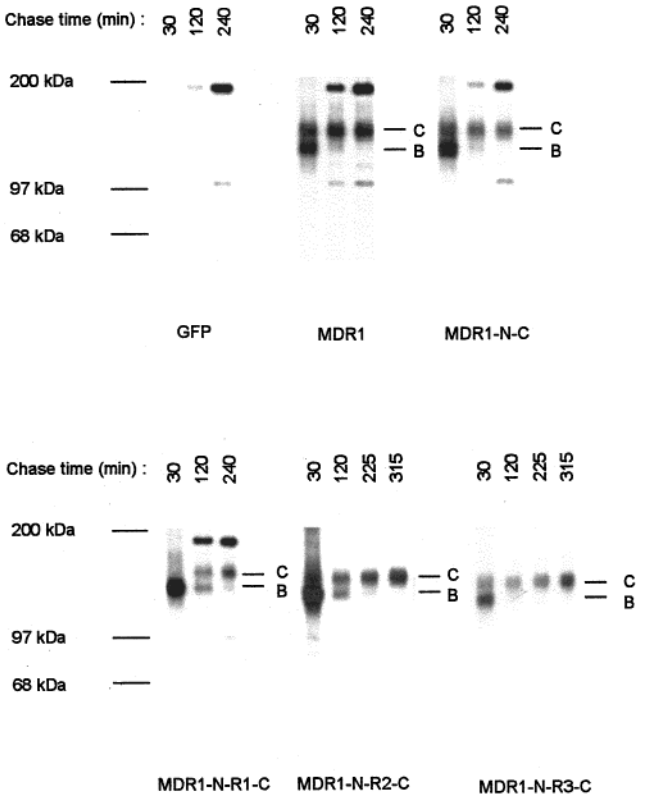

FIGURE 4: Pulse−chase and immunoprecipitation of MDR1 proteins from COS1 cells transiently expressing wild-type, deletion, and hybrid MDR1 proteins. Proteins were labeled with [^{35}S]methionine and [^{35}S]cysteine and chased for the indicated time periods, and MDR1 proteins were immunoprecipitated with an antibody directed against the C-terminal part of MDR1 (C219). The immunopurified proteins were separated on a 4−12% SDS gel and visualized by autoradiography. The core-glycosylated B-forms and the mature C-forms are indicated on the right for each construct. The names of the different expressed constructs are given (nomenclature of Figure 2); GFP indicates empty vector. The experiment was repeated with the same result.

780−830 (R3)] was deleted, a "wildtype" maturation pattern was obtained, whether replacement with the MDR1 linker domain was done or not.

Besides the immature and mature CFTR bands, a fragment with a molecular mass of 200 kDa was observed. This fragment was not present in all experiments and was also detected when MDR1 constructs were immunoprecipitated with an anti-MDR1 antibody (Figure 4) and when cells, transfected with a vector encoding GFP coding sequences alone, were immunoprecipitated with either antibody. Most probably, the protein is endogenously expressed in COS1 cells and binds to the Sepharose A beads and not to the monoclonal antibodies used for immunoprecipitation and, as a consequence, represents a nonspecific band.

2. Maturation of MDR1 Deletion and Hybrid Constructs. Wild-type MDR1 translation products are not subject to a high degree of degradation in the endoplasmic reticulum (Figure 4). Deletion of the linker domain (MDR1-N-C) slightly reduced the maturation efficiency of the MDR1 proteins, compared to wild-type MDR1. Replacement of the linker domain by the R domain fragment encompassing amino acids 651−830 (R1) resulted in a delay in maturation: after 30 min of chase, about half of the wild-type MDR1 translation products were already converted to the mature form, while all the translation products derived from the hybrid construct were still in their immature form. When hybrids with smaller parts of the R domain were used, the same delay in maturation was observed for R2 (amino acids 708−830; MDR1-N-R2-C); for the smallest fragment R3 (amino acids 780−830; MDR1-N-R3-C), the "wildtype" maturation pattern was found.

When immunoprecipitations were performed on the different hybrid constructs at later chase times, the intensity of the signal for the mature protein increased as did the total amount of signal present in the lane (compare signals after 120 and 240 min of chase for MDR1-R1 and after 225 and 315 min of chase for MDR1-N-R2-C and MDR1-N-R3-C in Figure 4), a feature that has been described by Cuppens et al. (*33*) for CFTR proteins too. This could indicate that during maturation and trafficking through the Golgi-stacks to the cell membrane, the glycosylated translation products attain folding intermediates that are not recognized by the antibody used for immunoprecipitation. Alternatively, the MDR1 hybrid proteins might be bound to other proteins, such as proteins needed for folding or trafficking, so that the epitope, that is recognized by the anti-MDR1 antibody, is masked. As a consequence, these intermediates would not be immunoprecipitated from the cell lysate by the antibody used, and would not be detected after autoradiography.

3. Electrophysiological Analysis of Maturing CFTR Deletion and Hybrid Constructs. To analyze the effect of the different R domain deletions and substitutions, maturing CFTR proteins lacking amino acids 708−830 (=R2) or 780−830 (=R3), or with these amino acids replaced by the linker domain of MDR1, were analyzed for their PKA- and PKC-dependent chloride transport activity using whole cell patch-clamp techniques.

3.1. Basal Chloride Transport Activity. The basal activity of the deletion constructs was not significantly different from wild-type CFTR when a small part of the R domain (R3) was deleted (CFTR-N3-C). Deletion of a bigger fragment of the R domain (R2) resulted in increased basal whole cell currents (CFTR-N2-C, $P = 0.026$; Figure 5; 6A,B). This basal current was, however, much lower than the "wildtype" PKA-induced whole cell current (Figures 5 and 6A), indicating that deletion of the R domain itself is not sufficient to fully activate the chloride channel. Replacement of both parts with the linker domain (CFTR-N2-L-C and CFTR-N3-L-C) also induced an increased basal activity (Figures 5 and 6A,B), indicating that an aberrant R domain structure might result in leakage of chloride ions through the channel pore.

3.2. Protein Kinase A Induced Chloride Transport Activity. When R2 or R3 were deleted (CFTR-N2-C and CFTR-N3-C), a significant reduction in the PKA-dependent whole cell chloride current, compared with wild-type CFTR, was observed (Figure 6A,C). When the MDR1 linker domain (CFTR-N2-L-C) substituted the biggest part of the R domain (R2), only a small increase in the cAMP-inducible chloride current was observed, compared to the deletion construct (CFTR-N2-C). Replacing R3 by the linker domain (CFTR-N3-L-C), which has a similar length as R3 and contains three in vitro used PKA sites (*34*), resulted in restoration of the PKA-induced whole cell currents to wild-type levels (Figures 5 and 6A,C).

3.3. Protein Kinase C Induced Chloride Transport Activity. PKC-stimulated whole cell chloride currents were similar for wild-type and deletion constructs (CFTR-N2-C and

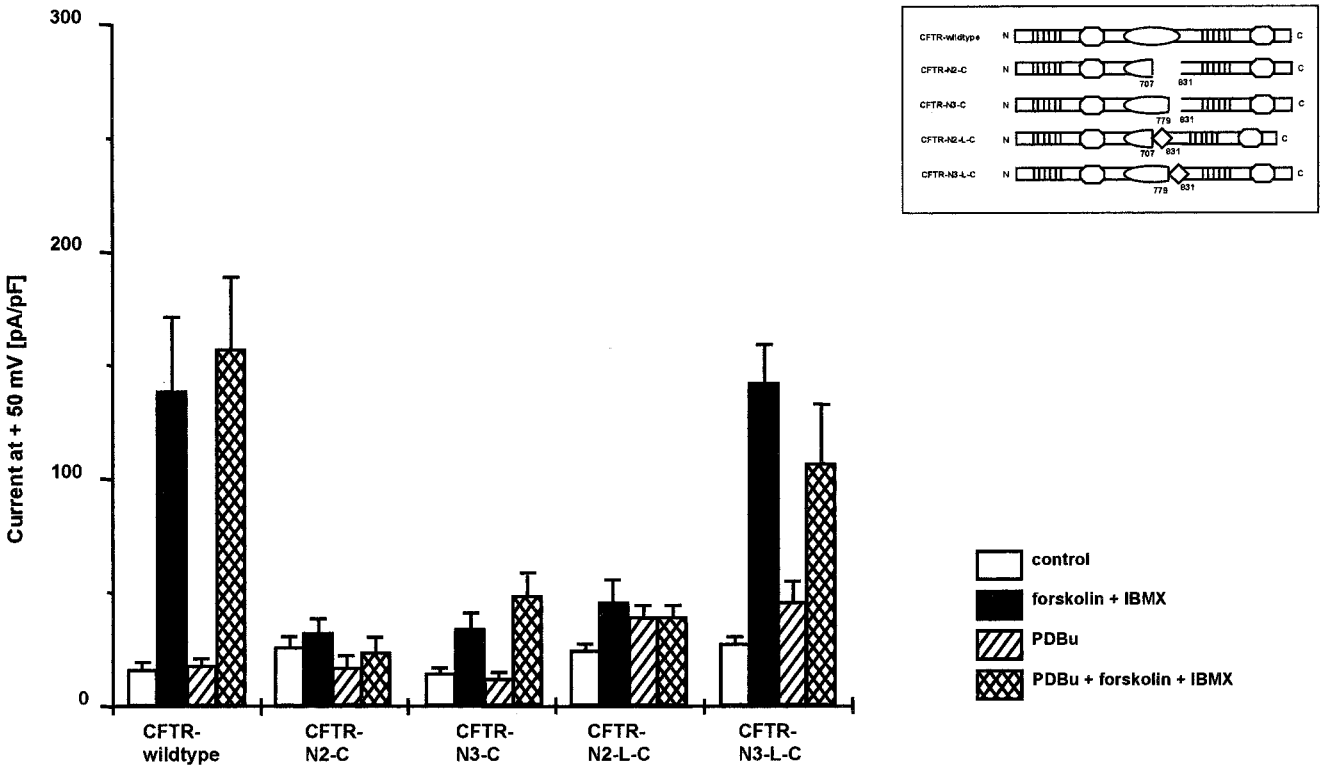

FIGURE 5: Summary of whole cell chloride currents measured before and after stimulation of COS1 cells, transfected with the different CFTR constructs, with forskolin/IBMX and/or PDBu. Pooled data were obtained from whole cell chloride currents measured, in wild-type CFTR or hybrid CFTR expressing cells, at +50 mV using the whole cell patch-clamp technique in the conditions indicated. Forskolin + IBMX indicates activation of protein kinase A, PDBu indicates activation of protein kinase C, and PDBu + forskolin + IBMX indicates prestimulation of protein kinase C by PDBu followed by stimulation of protein kinase A by forskolin and IBMX. At least 5 patches were measured for the different constructs in the different conditions. A significant difference ($P < 0.05$) was found for all CFTR-expressing cells tested for PKA activation, except for CFTR-N2-C, which showed only a small activation which could only be identified using the paired *t*-test. Only CFTR-N2-L-C and CFTR-N3-L-C showed significant activation by PDBu, when compared to the basal state. The structures of the different constructs analyzed are given in the inset.

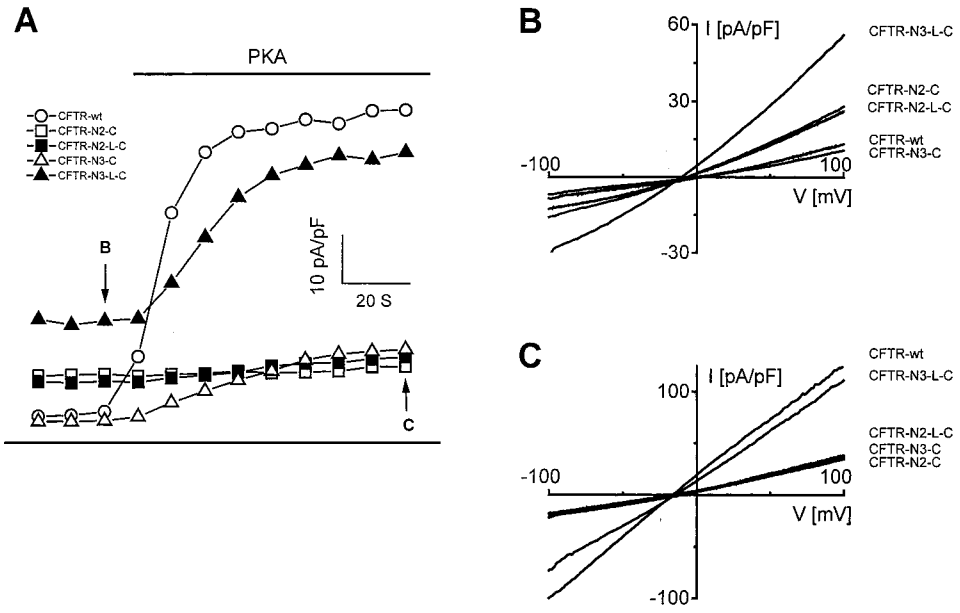

FIGURE 6: Activation of CFTR chloride currents by forskolin and IBMX. (A) Time course of whole cell chloride currents measured at +50 mV in wild-type and hybrid CFTR expressing cells. Forskolin and IBMX were added to the bath solution as indicated (PKA). (B) Basal current−voltage relationships drawn from the data collected from time point B, indicated in panel A. (C) Current−voltage relationships obtained after stimulation by forskolin and IBMX. The curve is drawn from the data collected from time point C, indicated in panel A.

CFTR-N3-C), indicating that PKC sites present in the deleted R domain parts are not important for chloride transport activity (Figure 7). When R2 or R3 were replaced by the MDR1 linker domain (CFTR-N2-L-C and CFTR-N3-L-C), a significant increase in PKC-dependent whole cell current was observed, compared to wild-type CFTR (Figure 7), indicating that the PKC consensus sites present in the linker domain are functional in the CFTR−MDR1 hybrid proteins.

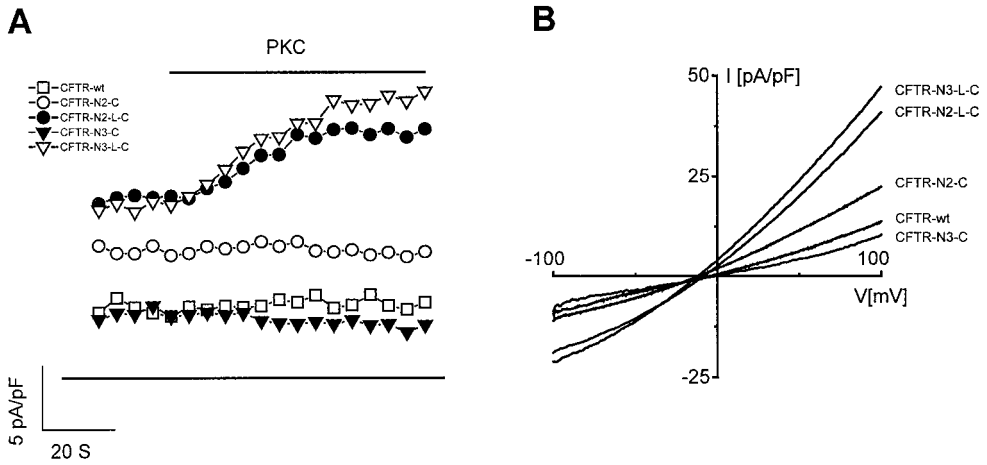

FIGURE 7: Activation of CFTR chloride currents by PDBu. (A) Time course of whole cell chloride currents measured at +50 mV in wild-type and hybrid CFTR expressing cells. PDBu was added to the bath solution as indicated (PKC). (B) Current−voltage relationships drawn from the data collected from the last time point shown in panel A.

These currents were, however, much lower than the PKA-induced wild-type ones (Figure 5).

3.4. Combined Effect of Protein Kinases A and C on Whole Cell Chloride Transport. Prestimulation of cells, transfected with the different constructs, with PDBu followed by PKA stimulation resulted in the appearance of whole cell chloride currents that were not significantly different from chloride currents measured after stimulation with forskolin and IBMX only (Figure 5). This indicates again that the PKC sites present in the CFTR R domain are not important for chloride transport activity and that phosphorylation of PKC consensus sites present in the MDR1 linker does not induce an additional activation of chloride currents passing through the hybrid CFTR channels.

DISCUSSION

Deletion of amino acids 651−830 (R1) of the R domain disrupts maturation of the CFTR translation product completely. Replacement of this fragment by the linker domain of MDR1 could only partially restore this defect, and certainly did not improve the maturation up to that of MDR1. Deletion of smaller parts of the R domain [aa 708−830 (R2) and aa 780−830 (R3)] had no influence on the maturation efficiency. Amino acids 708−830 could therefore form discrete domains that do not affect the overall folding of CFTR when they are deleted. The observation that CFTR, that lacks amino acids 651−830 (CFTR-N1-C), fails to mature might then be explained by the fact that amino acids 651−707 are part of another discrete domain which affects overall CFTR folding. Alternatively, only specific amino acids present in the fragment encompassing amino acids 651−707 might be indispensable for CFTR folding.

Replacement of the linker domain of MDR1 with various parts of the exon 13 R domain of the CFTR protein did not affect its overall maturation efficiency. The rate of maturation was, however, retarded when R1 or R2 were present. This indicates that sequences within R1 and R2 or that insertion of a bulkier cytosolic group as a whole might hamper the folding processes occurring in the endoplasmic reticulum. Deletion of the various exon 13 parts of CFTR did not result in an increase, and insertion in MDR1 did not result in a decrease, of the maturation efficiency of the resulting hybrid proteins, when compared to the respective wild-type proteins. This would suggest that not the R domain as such but rather sequences located outside the "R domain" or the overall structure of CFTR are important for this maturation deficiency.

Although the CFTR R domain is important for channel function, its precise way of action is still unknown. Deletion experiments performed by Rich et al. (*32*) indicate that the R domain forms a plug that covers the mouth of the pore and is repulsed from the pore when the R domain is phosphorylated. This hypothesis is supported by the profound effect of R domain mutations, that do not affect phosphorylation sites but do affect charged amino acids, on the activity of the CFTR chloride channel (*35*). The whole cell measurements performed on a construct deleted for amino acids 708−830 (=CFTR-N2-C) further support this hypothesis: the basal chloride current passing through the cell membrane is bigger for the deletion construct than for wild-type CFTR. Deletion of a smaller, more C-terminal part of the R domain (R3) was without effect. When both deletion constructs, CFTR-N2-C and CFTR-N3-C, were stimulated by forskolin and IBMX, the small increase in whole cell current was higher for CFTR-N3-C when compared to their respective basal currents. When the MDR1 linker domain replaced the deleted R domain parts, the degree of stimulation by PKA was also much higher for CFTR-N3-L-C than for CFTR-N2-L-C and even reached "wildtype" whole cell currents for CFTR-N3-L-C. These data indicate that sequences present in the fragment encompassing amino acids 708−779, and which are present in N3 but absent from N2, are necessary to close the channel in the basal state and, in an indirect way, are necessary to open the channel after PKA activation. This fragment contains four PKA consensus sites. One of these sites is phosphorylated in vivo [S737 (*36*)], and two are phosphorylated in vitro [S712 (*37*) and S768 (*38*)]. Wilkinson et al. (*14*) have studied the effect of phosphorylation of these sites on the activation state of the CFTR chloride channel. They found that activation was not influenced by phosphorylation of residue S712 but was inhibited by phosphorylation of residues S737 and S768. Phosphorylation of these sites might occur in the basal state and might be necessary to keep the channel closed. Deletion of this part will then result in an increased basal activity and could therefore explain the higher basal activity found

when R2 was deleted, compared to wild-type or CFTR deleted for R3. Phosphorylation of R3 by PKA might make the PKA consensus sites in fragment 708−779 accessible to phosphatases. Dephosphorylation of these inhibitory PKA sites might then result in conformational changes which are necessary to fully activate the chloride channel. Alternatively, this region might be important for full activation of CFTR by ensuring the accessibility of PKA consensus sites present in R3 to protein kinase A. It is also possible that this region is necessary for the transduction of conformational changes, introduced by phosphorylation of stimulatory PKA consensus sites located in the inserted MDR1 linker in the hybrid constructs or located in R3 in wild-type CFTR.

The MDR1 linker domain contains three consensus sites for protein kinase C, which are used in vitro and in vivo (*34*, *39*), and three for protein kinase A, which can be phosphorylated in vitro (*34*). The importance of these sites for MDR1 function is unknown. When R2 was replaced by the MDR1 linker domain, only a small increase in PKA-dependent chloride current was measured. When R3 was replaced by the linker, however, a restoration of the cAMP-dependent chloride current to "wildtype" levels was found. The linker domain and R3 are of similar length, and both contain three PKA consensus sites. This indicates that both domains might have similar structures and that the PKA sites present in the linker domain might be phosphorylated and able to stimulate the opening of the CFTR chloride channel in a similar way as those present in R3. This also indicates that not the specific amino acid sequences present in this domain are important but rather its overall structure and length, and the presence of PKA sites. This hypothesis is supported by the low conservation found, at the amino acid level, when CFTR sequences of the C-terminal part of the R domain of different species are aligned (*40*). Alternatively, the linker domain might not be phosphorylated itself, but its presence might facilitate the phosphorylation of consensus sites present in the remainder of the hybrid CFTR-N3-L-C protein.

The R domain contains two consensus sites for protein kinase C, S586 and S790. Phosphorylation of CFTR by PKC, however, does not induce chloride transport activity, indicating that phosphorylation of the R domain by PKC alone is not sufficient to open the chloride channel, as has been observed by Winpenny et al. (*17*). The different deletion constructs were also insensitive for PKC activation, but when the MDR1 linker domain substituted R2 or R3, activation of chloride transport could be observed. These results suggest different properties for the respective PKC sites. Both hybrid constructs induced similar PKC-dependent chloride transport activities that were much lower than "wildtype" PKA-induced whole cell chloride currents. When PKC stimulation of the different constructs was followed by PKA activation, no further increase in whole cell chloride currents occurred, compared to stimulation by PKA alone. This indicates that PKC does not enhance the PKA-stimulated activity of wild-type CFTR, in contrast to what has been observed earlier (*17*). These findings also indicate that phosphorylation of S661 of the MDR1 linker domain does not occur or that its phosphorylation does not enhance the activation state of the hybrid channels.

In summary, these studies indicate the R domain itself is not responsible for the inefficient maturation of CFTR found in the ER, but rather the overall structure of the protein. Further studies will be indispensable to elucidate the cause of this energy-wasting process. Not the precise sequence of the R domain, but rather its length and the presence and distribution of consensus sites for protein kinase A appear to determine the coupling of activation by cAMP to chloride transport through the pore of the CFTR chloride channel. Its precise way of action remains unresolved.

ACKNOWLEDGMENT

We thank Jan Eggermont, who kindly provided pCINeo/IRES-GFP, P. Sonneveld for receiving *MDR1*cDNA, and Transgene S.A., Strasbourg, France, who generously provided the pTG5960 vector.

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BI991520D