Control of Cystic Fibrosis Transmembrane Conductance Regulator Expression by BAP31*

Expression of the cystic fibrosis transmembrane conductance regulator (CFTR), which is a Cl− channel and a regulator of other ion channels (1), is usually 1% or less of the amount of wtCFTR present in the native tissue and is probably higher in Xenopus oocytes (2, 14). The enzymes and compartments responsible for degradation of CFTR are part of the ER quality control system of secretory proteins (8). Binding of CFTR to the ER membrane chaperone calnexin (9) and the cytosolic chaperone Hsp70 or Hsp90 (10, 11) has been demonstrated. Small amounts of [ΔPhe508]CFTR that escape proteolysis is trafficked to the cell membrane, where it functions as cAMP-regulated Cl− channel (12, 13). This is usually 1% or less of the amount of wtCFTR present in the native tissue and is probably higher in Xenopus oocytes (2, 14).

Recently, several proteins have been identified which bind to BAP31, and related proteins play a role in vesicular transport and control anterograde transport of certain membrane proteins such as cellubrevin (16, 17). We therefore examined in the present study whether BAP31 also affects expression of CFTR. We were particularly interested to see whether expression of [ΔPhe508]CFTR can be influenced by manipulation of the expression of BAP31. The data presented here demonstrate that BAP31 inhibits both expression of wtCFTR as well as [ΔPhe508]CFTR and suggest that BAP31 is participating in formation of the quality control system.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfection with BAP31 Antisense Oligonucleotides—CHO cells expressing either wtCFTR or [ΔPhe508]CFTR (kindly provided by Dr. X.-B. Chang and Prof. J. Riordan, Mayo Clinic Scottsdale, Scottsdale, AZ) were cultured in a minimum essential medium supplemented with 8% fetal bovine serum, 50 μmol/liter methotrexate, penicillin (100 units/ml) and streptomycin (100 μg/ml) (Life Technologies, Germany) in a humidified atmosphere with 5% CO2 (18). Human bronchial epithelial cells (16HBE14o−) and mouse epithelial-collecting duct cells (M1) were cultured as described previously (19, 20). About 5 × 106 CHO cells expressing [ΔPhe508]CFTR were electroporated in the presence of 12 nmol/liter (40 μg/ml) of a plasmid encoding the enhanced green fluorescent protein (pEGFP-c1; CLONTECH, Germany) and about 0.5 μmol/liter (3.5 μg/ml) phosphorothioated (stabilized) oligonucleotides antisense to the first 20 bases of the BAP31 coding sequence. Control cells were transfected with EGFFP and missense oligonucleo-

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2 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; CHO, Chinese hamster ovary cells; GST, glutathione S-transferase; IBMX, 3-isobutyl-1-methylxanthine; EGFP, enhanced green fluorescent protein; BAP, B cell antigen receptor-associated protein; wt, wild-type; HBE, human bronchial epithelial cells; M1, mouse epithelial-collecting duct cells.
BAP31 Controls Expression of CFTR

BAP31 Controls Expression of CFTR and Is Colocalized with CFTR in CHO Cells—ΔPhe508CFTR does not mature properly and is therefore excluded from the glycosylation pathway. Thus, ΔPhe508CFTR appears as a lower molecular weight band on an SDS gel when compared with wtCFTR. We analyzed expression of wtCFTR and ΔPhe508CFTR in CHO cells stably transfected with either wtCFTR or ΔPhe508CFTR (4). Mature and fully glycosylated CFTR of molecular weight of about 180 kDa is detected in wtCFTR-expressing cells whereas ΔPhe508CFTR appears as an immature precursor of a lower molecular weight of about 150 kDa (Fig. 1A). CHO cells do also show expression of BAP31 as indicated by Western blot analysis (Fig. 1A). Both wtCFTR- and ΔPhe508CFTR-expressing cells were transfected with BAP31 antisense oligonucleotides (+ AS), which largely reduced expression of BAP31 when compared with control cells (−AS). Parallel to the reduced expression of BAP31, we detected an increase in both expression of wtCFTR as well as ΔPhe508CFTR, suggesting that expression of CFTR is controlled by BAP31. Experiments were carried out at least in triplicates but generally 5–7 times. Expression of BAP31 was also detected in various cultured cell types from pancreas (CFPAC), collecting duct (M1), colon (HT29 and T84) and airways (CFBE, 16HBE14o−, 9HTE), although levels of BAP31 expression varied significantly between the different cell lines. In all these cell lines, CFTR is expressed endogenously (data not shown). In mouse collecting duct cells (M1) and human bronchial epithelial cell lines (16HBE14o−; HBE), expression of BAP31 was blocked by antisense treatment. Reduced expression of BAP31 was paralleled by enhanced expression of CFTR (Fig. 1B). These data suggest that BAP31 also controls expression of endogenous CFTR in airways and collecting duct of the kidney.

Because Western blot analysis suggested that BAP31 is involved in the control of synthesis and maturation of CFTR, we performed immunofluorescence stain of both CFTR and BAP31 (Fig. 2). The cells showed a perinuclear staining for BAP31 (Texas red fluorescence) and were also significantly stained for wtCFTR (DTAF green fluorescence). The overlay (yellow fluorescence) of both pictures shows colocalization of both proteins (Fig. 2). This data suggest that both CFTR and BAP31 are colocalized in the ER of CHO cells and thus support the idea of BAP31 controlling...
maturation or trafficking of CFTR.

Inhibition of BAP31 Expression Enhances Cl⁻ Conductance and Recovers Cl⁻ Channel Activity in Cells Expressing ΔPhe508CFTR—To examine whether BAP31 also affects functional expression of CFTR, we analyzed cells transfected with BAP31 antisense as well as control cells in a 36Cl⁻ efflux assay. To that end, electroporated CHO cells were grown on 35-mm culture dishes and loaded with 36Cl⁻. Subsequently, 36Cl⁻ efflux was measured in the absence or presence of forskolin (10 μM/liter). As shown in Fig. 3, 36Cl⁻ efflux was continuously declining in the absence of forskolin (dashed lines). In wtCFTR-expressing CHO cells, stimulation with forskolin largely enhanced 36Cl⁻ efflux, indicating activation of a CFTR Cl⁻ conductance, whereas no 36Cl⁻ efflux was activated in ΔPhe508CFTR-expressing CHO cells. However, in BAP31 antisense-treated cells expressing ΔPhe508CFTR, forskolin was able to induce a small but significant 36Cl⁻ efflux, and 36Cl⁻ efflux in wtCFTR-expressing cells was augmented (solid lines, filled circles). These data suggest that blocking of BAP31 expression enhances CFTR expression and leads to a residual Cl⁻ channel activity in ΔPhe508CFTR-expressing CHO cells.

We further cotransfected wtCFTR or ΔPhe508CFTR-expressing CHO cells with a 40-fold molar excess of BAP31 antisense oligonucleotides and the expression plasmid for green fluorescent protein (pEGFP-C1). Control cells were transfected with pEGFP-c1 only. EGFP fluorescence was monitored during subsequent patch clamp experiments and was used as an indicator for successful transfection. Only fluorescent-labeled cells were used for patch clamp experiments. Activation of wtCFTR was studied initially in wtCFTR-expressing cells. Upon stimulation with IBMX (100 μM/liter) and forskolin (10 μM/liter), a large whole cell conductance was activated and the cell membrane voltage (Vm) was depolarized (Fig. 4, A and B, upper traces). Partial replacement of extracellular Cl⁻ by impermeable gluconate (30Cl⁻) partially blocked the activated whole cell conductance and further depolarized Vm, indicating activation of a whole cell Cl⁻ conductance. In the absence of BAP31 antisense, [ΔPhe508]CFTR-expressing cells did not show a response to any of the above maneuvers (Fig. 4, A and B, middle trace). However, in BAP31 antisense-incubated cells, forskolin and IBMX were able to activate a residual Cl⁻ conductance as indicated by changes in whole cell conductance and Vm (Fig. 4, A and B, lower trace). Fig. 5 summarizes the effects of IBMX and forskolin and 30Cl⁻ on whole cell conductances and membrane voltages and clearly indicates a significant cAMP-activated whole cell Cl⁻ conductance in CHO cells expressing [ΔF508]CFTR after blocking expression of BAP31.

Coexpression of BAP31 and CFTR in Xenopus Oocytes—We further examined how coexpression of BAP31 affects activation of wtCFTR in oocytes of X. laevis. Fig. 6A shows whole cell recordings obtained from oocytes expressing only wtCFTR or coexpressing wtCFTR together with BAP31. The corresponding I/V curves are shown in Fig. 6B. The tracings, I/V curves, and the summary (Fig. 6C) of this series of experiments clearly show that coexpression of BAP31 attenuates significantly the activation of a CFTR Cl⁻ conductance in Xenopus oocytes. Injection of a non-translated missense-cRNA did not affect CFTR currents, suggesting that decrease in CFTR is caused by expression of BAP31 rather than nonspecific effects because of additional cRNA injection (data not shown).

To further elucidate the impact of BAP expression on CFTR Cl⁻ conductance, we coexpressed a BAP31 variant lacking the last C-terminal 24 nucleotides. This truncated BAP31 version lacks the C-terminal KKEE amino acid motif, which is known as an ER retrieval sequence (consensus sequence KXXX) and which may also have a function as a transport or internalization signal (26, 27). The ability to inhibit CFTR expression in Xenopus oocytes seems to rely on the presence of the KXXX motif, because BAP31-KXXX was unable to decrease cAMP-activated CFTR Cl⁻ conductance (Fig. 7). Transfection with BAP31 carrying the KXXX may cause an ER overload and may thus cause stress to the cells (28). To exclude artificial effects on CFTR Cl⁻ conductance caused by possible ER stress, we coexpressed CFTR together with the adenovirus protein E3/19K. E3/19K has been demonstrated to accumulate in ER membranes and to cause cells stress, thereby activating the transcription factor NFκB (28). However, ER stress does not seem the cause for reduced CFTR expression in Xenopus oocytes, because E3/19K exerted no inhibitory effects on CFTR Cl⁻ conductance (Fig. 7). Finally, BAP29, a protein homologous to BAP31 was coexpressed together with CFTR and slightly but not significantly attenuated activation of CFTR upon increase in intracellular cAMP. When coexpressed together with BAP31, heterodimerization of BAP29 and BAP31 is likely to occur (16). The putative BAP31/BAP29 heterodimer was also able to inhibit expression of CFTR Cl⁻ conductance, as shown in Fig. 7. Taken together, these data strongly suggest regulation of CFTR expression by BAP31, which might be part of the quality control system.

**Fig. 1.** Western blot analysis of the expression of wtCFTR, [ΔF508]CFTR and BAP31 in CHO, M1, and HBE cells. Equal amounts of protein (20 μg) were isolated from CHO cells, grown in the presence of a BAP31 antisense oligonucleotide (+AS) or missense oligonucleotide (−AS), and from M1 and HBE cells growing either in the presence or absence of BAP31-antisense, and were separated on SDS gels. When grown in the presence of antisense nucleotides, expression of BAP31 was largely reduced in CHO cells (A) and the epithelial cell lines M1 and HBE (B). The decrease in BAP31 expression was paralleled by an increase in CFTR expression in CHO cells, expressing CFTR exogenously (A), and the epithelial cell lines M1 and HBE, expressing CFTR endogenously (B). Individual experiments were carried out 3–7 times.
The present data suggest that the putative integral membrane protein BAP31 interferes with expression of CFTR in heterologous expression systems. BAP31 was identified initially in the murine myeloma cell line J558L and was shown later to be expressed in various cell types (15). In the present study, we detected expression of BAP31 in CHO cells and also in various types of human epithelial cell lines derived from colon, pancreas, kidney collecting duct, and airways (data not shown). Both BAP29 and BAP31 contain three stretches of hydrophobic amino acids, suggesting that these proteins are multiple spanning transmembrane proteins (16). When BAP31 expression was blocked by antisense oligonucleotides in CHO cells, expression of both wtCFTR or \[^508\text{CFTR}\] seemed to be enhanced. The amount of mature \[^508\text{CFTR}\] protein was enhanced when expression of BAP31 was inhibited in CHO cells, expressing exogenous wtCFTR. Our data show that this effect is not limited to cells overexpressing CFTR. In airway epithelial and collecting duct cells, inhibition of BAP31 expression induced enhanced expression of endogenous CFTR. Expression of mature \[^508\text{CFTR}\] in CHO cells after BAP31-antisense oligonucleotide treatment was significantly higher than in control cells, indicating a regulatory role for BAP31 in CFTR expression.
Golgi (16, 17). It has also been demonstrated to function as a residual proteins of the ER, which cycle between ER and the CFTR only; (), number of experiments.

pared with whole cell conductance measured in oocytes expressing differences (Student’s paired t-test); #, significant difference from control (paired Student’s t-test).

ared with cRNA encoding (i) BAP31 lacking the C-terminal KK XX motif (ii) the ER-localized transmembrane protein E3/19k; (iii) BAP29, and (iv) BAP29 together with BAP31. *, significant difference from control (unpaired Student’s t-test).

FIG. 6. Impact of BAP31 on the activation of CFTR Cl− conductance in Xenopus oocytes. A, whole cell currents activated by IBMX and forskolin (1 nmol/liter and 2 μmol/liter) in CFTR expressing oocytes and impact of the coexpression of BAP31. B, I/V curves for the IBMX- and forskolin-activated whole cell currents in water-injected control oocytes and oocytes expressing CFTR (CFTR-BAP31) or coexpressing CFTR and BAP31 (CFTR+BAP31). C, summary of the whole cell conductances activated by IBMX and forskolin in oocytes expressing CFTR or coexpressing CFTR and BAP31. (), number of experiments; *, significant difference from control (paired Student’s t test); #, significant difference from CFTR-BAP31 (unpaired Student’s t test).

FIG. 7. Summary of whole cell conductances activated by IBMX/forskolin in wtCFTR-expressing oocytes. Oocytes were coinjected with cRNA encoding (i) BAP31 lacking the C-terminal KKKX motif (+BAP31-KKKX), (ii) the ER-localized transmembrane protein E3/19k, (iii) BAP29, and (iv) BAP29 together with BAP31. *, significant differences (Student’s paired t test); #, significant difference when compared with whole cell conductance measured in oocytes expressing CFTR only; (), number of experiments.

The experiments performed in Xenopus oocytes supply further evidence for the impact of BAP31 on processing and expression of CFTR. Interestingly, coexpression of both BAP29 and BAP31 was even more efficient in inhibiting CFTR expression, compared with the effects of solely expression of BAP31. Thus, CFTR may interact with BAP31 homodimers or heterodimers of BAP31 and BAP29, which have been reported previously (16). The present data further stress the importance of the KKXX motif at the C-terminal end of BAP31, because no inhibition of CFTR conductance was observed in the absence of this motif. The KKXX motif is an ER retention signal for residual proteins of the ER, which cycle between ER and the Golgi (16, 17). It has also been demonstrated to function as a transport signal (30). KKXX-carrying proteins bind to COP proteins and are involved in the retrograde transport from the cis Golgi to the ER (31, 32). In addition, the KKXX motif may also serve as an internalization and endocytosis signal (27). The KKXX motif seems to be crucial for the inhibitory effects of BAP31 on CFTR. As mentioned above, the KKXX motif at the C-terminal end of BAP31 may lead to cell stress, activation of NFκB, and eventually cell death by apoptosis (28, 33). In fact, BAP31 probably takes part in the control of programmed cell death (34, 35). Although we cannot completely rule out a possible nonspecific effect of BAP31 on CFTR expression, we were able to show that expression of the protein E3/19k that binds very tightly to the ER and induces ER overload (28) does not interfere with expression of CFTR in Xenopus oocytes.

Interestingly, it has been shown recently that virus maturation in the ER is controlled by an KKXX ER retrieval signal and that viruses lacking the envelope glycoprotein encoding the KKXX motif have a higher chance for budding at the plasma membrane (36). The present results suggest that trafficking or maturation of a variety of transmembrane proteins might be controlled by BAP31, including CFTR. However, it is unlikely that BAP31 affects expression of all membrane proteins, because we did not detect an increased expression for the α-subunit of the Na+/K+-ATPase in BAP31-AS treated HBE or M1 cells (data not shown). One function of BAP31 could be that it participates in formation of the quality control mechanism of membrane protein synthesis, which was postulated for CFTR (37). It is now well known that biosynthesis of CFTR is strictly controlled by this mechanism, resulting in degradation of most of the wtCFTR and basically all of ΔPhe508CFTR (3). Because ΔPhe508CFTR is by far the most frequent mutation causing cystic fibrosis, interfering with the expression or function of BAP31 in epithelial cells could be a new way to circumvent the Cl− channel defect in cystic fibrosis.

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