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Evidence for phosphorylation of serine 753 in CFTR using a novel metal-ion affinity resin and matrix-assisted laser desorption mass spectrometry

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Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) gene encodes an apical membrane Cl^- channel regulated by protein phosphorylation. To identify cAMP-dependent protein kinase (PKA)-phosphorylated residues in full-length CFTR, immobilized metal-ion affinity chromatography (IMAC) was used to selectively purify phosphopeptides. The greater specificity of iron-loaded (Fe^{3+}) nitrilotriacetic (NTA) Sepharose compared to iminodiacetic acid (IDA) metal-chelating matrices was demonstrated using a PKA-phosphorylated recombinant NBD1-R protein from CFTR. Fe^{3+}-loaded NTA Sepharose preferentially bound phosphopeptides, whereas acidic and poly-His-containing peptides were co-purified using the conventional IDA matrices. IMAC using NTA Sepharose enabled the selective recovery of phosphopeptides and identification of phosphorylated residues from a complex proteolytic digest. Phosphopeptides from PKA-phosphorylated full-length CFTR, generated in Hi5 insect cells using a baculovirus expression system, were purified using NTA Sepharose. Phosphopeptides were identified using matrix-assisted laser desorption mass spectrometry (MALDI/MS) with post-source decay (PSD) analysis and collision-induced dissociation (CID) experiments. Phosphorylated peptides were identified by mass and by the metastable loss of HPO_3 and H_3PO_4 from the parent ions. Peptide sequence and phosphorylation at CFTR residues ^{660}Ser, ^{737}Ser, and ^{795}Ser were confirmed using MALDI/PSD analysis. Peptide sequences and phosphorylation at CFTR residues ^{700}Ser, ^{712}Ser, ^{768}Ser, and ^{813}Ser were deduced from peptide mass, metastable fragment ion formation, and PKA consensus sequences. Peptide sequence and phosphorylation at residue ^{753}Ser was confirmed using MALDI/CID analysis. This is the first report of phosphorylation of ^{753}Ser in full-length CFTR.

Keywords: CFTR; mass spectrometry; phosphorylation

The cystic fibrosis transmembrane conductance regulator gene encodes an apical membrane Cl^- channel (Riordan et al., 1989). Expression of CFTR results in increased Cl^- conductance following stimulation with cAMP agonists (Rich et al., 1990; Anderson et al., 1991; Bear et al., 1991; Drumm et al., 1991; Tabcharani et al., 1991). Chloride channel activity is proposed to be regulated by phosphorylation of the R domain (Berger et al., 1991, 1993; Cheng et al., 1991; Tabcharani et al., 1991) and by the interaction of ATP with the nucleotide binding folds (Anderson et al., 1991; Anderson & Welsh, 1992). It has been shown that Ser residues 660, 700, 737, 768, 795, and 813 are phosphorylated in vivo after stimulation of cAMP-dependent PKA (Cheng et al., 1991; Picciotto et al., 1992; Chang et al., 1993). However, mutagenesis of all 10 predicted dibasic PKA phosphorylation sites in CFTR (10Ser-Ala mutant CFTR) did not completely abolish stimulated Cl^- conductance (Chang et al., 1993). Phosphorylation of residue ^{753}Ser was identified as a major contributor to the remaining Cl^- conductance in the 10Ser-Ala mutant CFTR molecule (Seibert et al., 1995). Whether ^{753}Ser regulates the activity of wild-type (nonmutated) CFTR is not known.

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; CID, collision-induced dissociation; CZE, capillary zone electrophoresis; C_{18}, octadecyl silica; ESI/MS, electrospray ionization mass spectrometry; IDA, iminodiacetic acid; IMAC, immobilized metal-ion affinity chromatography; LC-ESI/MS, liquid chromatography-ESI/MS; MALDI/MS, matrix-assisted laser desorption MS; $[M+H]^+$, protonated molecular mass; NBD1-R, recombinant protein consisting of the first nucleotide binding domain (NBD) and the regulatory domain (R) of CFTR (residues 404–830) with a His_6-tag; Ni-NTA, nickel-nitrilotriacetic acid; NTA, nitrilotriacetic acid; PKA, cAMP-dependent protein kinase A; PSD, post-source decay; PTH, phenylthiohydantoin; RP/HPLC, reversed-phase HPLC.

This study was undertaken to determine the sites of CFTR phosphorylation using matrix-assisted laser desorption mass spectrometry. Phosphopeptides are identified as masses with 80-Da increments greater than the predicted peptide molecular weights (Yip & Hutchens, 1992; Craig et al., 1994; Liao et al., 1994; Annan & Carr, 1996). The analysis of phosphoserine-containing peptides by MALDI/MS in reflectron mode is facilitated by the formation of metastable fragments from the losses of HPO_3 and H_3PO_4 (Annan & Carr, 1996). However, MALDI/MS requires that individual fractions from either HPLC or CZE be collected, dried, and crystallized with matrix and analyzed individually. Also, separating the large numbers of peptides into distinct fractions by RP/HPLC for a large protein ($M_r \sim 180$ kDa) such as CFTR is impractical. Thus, phosphopeptides must be isolated before MALDI/MS.

The use of immobilized metal-ion affinity chromatography to purify phosphopeptides (Michel & Bennet, 1987; Flotow et al., 1990; Scanff et al., 1991; Muszynska et al., 1992; Watts et al., 1994) has been proposed to increase the usefulness of both MALDI/MS and liquid chromatography-electrospray ionization mass spectrometry for structural studies of protein phosphorylation. However, disadvantages of the presently used affinant, Fe^{3+}-iminodiacetic acid, are that binding to acidic peptides can occur (Muszynska et al., 1992) and incomplete iron loading can result in the co-purification of His- and Lys-containing peptides (Scanff et al., 1991).

In this report, we show that an Fe^{3+}-loaded quadradentate metal-chelator, nitrilotriacetic acid (Hochuli et al., 1987), binds with high specificity to phosphopeptides from a tryptic digest of the recombinant NBD1-R domains of CFTR. In contrast, when either Fe^{3+}-loaded IDA Sepharose or IDA POROS™ resin was used, a significantly greater number of acidic and poly-His peptides co-purified with phosphopeptides. This novel IMAC-MALDI/MS approach was then applied to the study of PKA-phosphorylated full-length CFTR. Phosphorylation of Ser residues 660, 700, 712, 737, 768, 795, and 813 was demonstrated using peptide mass, metastable fragment ion formation, and post-source decay analysis. Phosphorylation at Ser residue 753 was also found using peptide mass, metastable fragment ion formation, and collision-induced dissociation analysis.

422*
MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGS[404]GFGELFEKAKQNNNNRKTSNGDDSLF
489
FSNFSLLGTPVLKDINFKIERGQLLAVAGSTGAGKTSLLMMIMGELEPSEGKIKHSGRISFCS
519
QFSWIMPGTIKENIIFGVSYDEYRYRSVIKACQLEEDISKFAEKDNIVLGEGGITLSGGQRA
557
RISLARAVYKDADLYLLDSPFGYLDVLTEKEIFESCVCKLMANKTRILVTSKMEHLKKADKIL
660* 670*
ILNEGSSYFYGTFSELQNLQPDFSSKLMGCDSFDQFSAERRNSILTETLHRFSLEGDAPVSW
700* 712* 737*
TETKKQSFKQTGEFGEKRKNSILNPINSIRKFSIVQKTPLQMNGIEEDSDEPLERRLSLVP
753 768* 795*
DSEQGEAILPRISVISTGPTLQARRRQSVLNLMTHSVNQGQNIHRKTTASTRKVSLAPQANLTEL
813*
DIYSRRLSQETGLEISEEINEEDLK[830]

Fig. 1. Phosphorylation consensus sites of the NBD1-R domain of CFTR. The His_6-tagged NBD1-R domain protein construct is shown (CFTR residues 404–830). Protein kinase A consensus sequences are underlined and sites of phosphorylation identified previously by ESI/MS (Townsend et al., 1996) are marked by an asterisk.

Results

The NBD1-R domain of CFTR has 13 Ser-containing consensus sequences for PKA phosphorylation, with at least one Arg residue (Fig. 1), that are expected to have a "high phosphorylation" probability (Kemp et al., 1977). There are eight dibasic and five monobasic sites. It was shown previously that all the dibasic sites in the NBD1-R domain were phosphorylated by PKA (Townsend et al., 1996).

To isolate phosphopeptides specifically from full-length CFTR, three different IMAC matrices (IDA Sepharose, IDA POROS™ resin, and NTA Sepharose) were evaluated using the phosphorylated NBD1-R protein. Peptide desalting prior to IMAC was necessary because the efficiency of phosphopeptide binding appeared to decrease when nondesalted peptide mixtures were analyzed (data not shown). This may be due to the presence of ATP, urea, and other salts. ATP is known to bind to Fe^{3+}-loaded matrices (Muszynska et al., 1992) and, when present in large excess over phosphopeptide, may compete with and/or block phosphopeptide binding. The peptides that bound to the IMAC columns and were eluted at high pH were subjected to C_{18} RP/HPLC. Figure 2 shows the chromatograms obtained from the peptides bound by NTA Sepharose (Fig. 2A) and IDA Sepharose (Fig. 2B). IDA POROS™ resin and IDA Sepharose results were similar (data not shown). Additional NBD1-R peptides bound to the IDA Sepharose; there were 11 peaks eluting with $t_r < 30$ min with the IDA Sepharose (com-

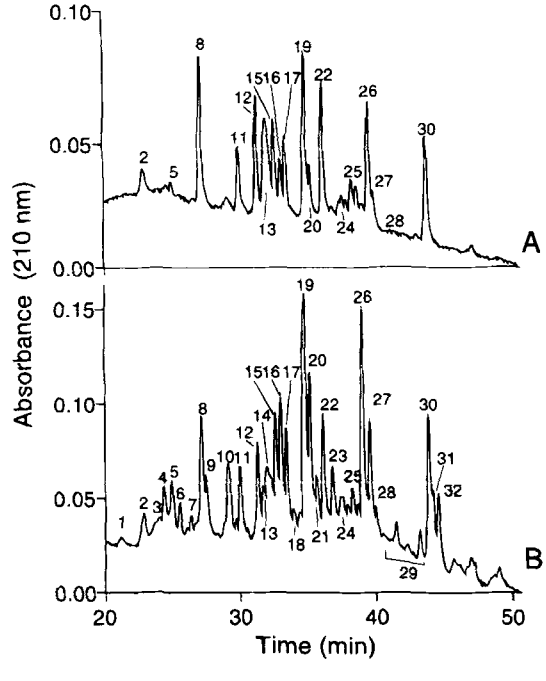

Fig. 2. RP/HPLC of NBD1-R tryptic peptides purified by IMAC. NBD1-R tryptic peptides from either NTA Sepharose (**A**) or IDA Sepharose (**B**) were injected onto the C_{18} RP/HPLC column and eluted isocratically with 2% solvent B for 10 min followed by a linear gradient of 2–50% solvent B over 50 min. The eluant was monitored by on-line UV absorbance detection at 210 nm. The collected peaks are numbered.

pared to 4 for the NTA matrix) and an increase in the areas of peaks 20–29 and 31–32. The remaining peaks were seen in both Figure 2A and B.

To determine whether the NTA Sepharose bound all of the expected phosphopeptides in the tryptic digest of phosphorylated NBD1-R (Townsend et al., 1996), the RP/HPLC peak fractions were analyzed by MALDI/MS. The spectra obtained were analyzed for phosphopeptides by searching for phosphate moiety increments (80 Da) to the theoretical masses of the nonphosphorylated tryptic peptides. A representative analysis for one phosphopeptide-containing fraction (Fig. 2, peak 19) is described below.

The MALDI/MS spectrum obtained in reflectron mode for peak 19 (Fig. 2A) is shown in Figure 3. The ion at *m/z* 1,961.2 corresponded to the $[M+H]^+$ of the phosphorylated ^{735}Arg-Arg^{751} peptide. Ions at *m/z* 1,203.1, 1,825.4, 1,848.3, 1,865.6, 1,883.1, and 2,162.3 were also observed. Only masses corresponding to the ions at *m/z* 1,961.2 and 2,162.3 were seen when this fraction was analyzed in linear mode (data not shown). This result demonstrated that these were parent ion masses and the remaining ions were likely generated from metastable fragmentation. The assignment of the ion *m/z* 1,961.2 as the parent ion for the signals at *m/z* 1,203.1, 1,825.4, 1,848.3, 1,865.6, and 1,883.1 was demonstrated by the use of a Bradbury–Nielson ion-gate in the first field-free region of the mass spectrometer (post-source and pre-reflector), which allowed analysis of ions in a selected mass window. When the ion at *m/z* 1,961.2 was selected, ions at *m/z* 1,203.1, 1,825.4, 1,848.3, 1,865.6, and 1,883.1 were still observed. This experiment also showed that these five ions were not metastable fragments from the ion at *m/z* 2,162.3. The ions at *m/z* 1,883.1 and 1,865.6 corresponded to the loss of HPO_3 and the β-elimination of phosphoric acid (H_3PO_4), respectively. These fragments were formed post source, because they were not detected at precisely 80- and 98-Da increments, respectively, from the parent phosphopeptide ion. This mass inaccuracy due to metastable fragment ion formation was reported to occur for Asp-Pro and Asp-Xxx peptide bonds that also undergo cleavages in MALDI/MS (Yu et al., 1993). The product formed from the loss of H_3PO_4 was the most abundant ion

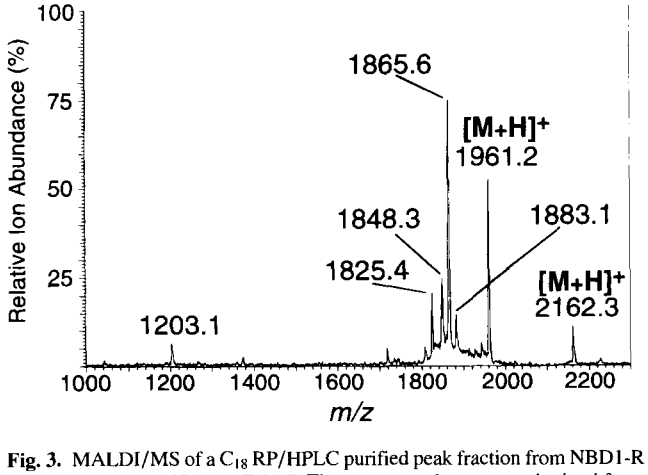

Fig. 3. MALDI/MS of a C_{18} RP/HPLC purified peak fraction from NBD1-R tryptic peptides purified by IMAC. The spectrum shown was obtained from peak 19 (Fig. 2A). Tryptic digests from phosphorylated NBD1-R that bound to either NTA Sepharose or IDA Sepharose were subjected to C_{18} reverse-phase chromatography as described in the Materials and methods. The collected peaks were analyzed by MALDI/MS in reflectron mode as positive ion data. The matrix was α-cyano-4-hydroxycinnamic acid.

detected for this and all other phosphopeptides examined in this study. The amount of metastable fragmentation appeared to be dependent on phosphopeptide sequence because the relative abundance of parent, parent-HPO_3, and parent-H_3PO_4 ions were not consistent for all phosphopeptides examined. The formation of breakdown products from the β-elimination of phosphoric acid and from the loss of HPO_3 is characteristic of phosphoserine-containing peptides, in agreement with published observations (Annan & Carr, 1996) for both phosphoserine and phosphothreonine residues.

Phosphopeptides were analyzed using MALDI/MS in both reflectron and linear mode. When phosphoserine-containing peptides were examined in linear mode, metastable fragments were not observed because both parent and daughter ions were detected at the same time. In one instance, the parent ion of the ^{752}Ile-Arg^{764} phosphopeptide from NBD1-R was only observed in linear mode when analyzed using a TofSpec SE mass spectrometer because the parent ion underwent total metastable decay in the mass spectrometer. This phosphopeptide would not have been observed if only reflectron mode analysis had been used. However, this same phosphopeptide, when purified from full-length CFTR, did not undergo complete metastable fragmentation when analyzed using an AutoSpec 5000 mass spectrometer. Because a different matrix solution was used, it is possible that the MALDI matrix may affect the observed metastable fragmentation. Therefore, reflectron and linear modes are complementary in the MALDI/MS analysis of phosphopeptides.

The peptide at *m/z* 1,961.2 that corresponded to the $[M+H]^+$ of the phosphorylated ^{735}Arg-Arg^{751} peptide was subjected to MALDI/PSD analysis (Fig. 4). Ions corresponding to the $[M+H]^+$-80 (*m/z* 1,881.7) and $[M+H]^+$-98 (*m/z* 1,863.5) were observed from the loss of HPO_3 and the β-elimination of phosphoric acid, respectively. Due to preferential charge retention on Arg residues, both **b** (N-terminal) and **y** (C-terminal) ions were observed. Ions corresponding to $\mathbf{y}_1$ (*m/z* 175.0), $\mathbf{y}_2$ (*m/z* 272.3), $\mathbf{y}_5$ (*m/z* 569.7), $\mathbf{y}_8$ (*m/z* 884.1), $\mathbf{y}_{10}$ (*m/z* 1,100.8), $\mathbf{y}_{12}$ (*m/z* 1,312.9), and $\mathbf{y}_{14}$ (*m/z* 1,524.9) were seen. The absence of $\mathbf{y}_n$-80 or $\mathbf{y}_n$-98 (**n** equals 1–14), demonstrated the absence of phosphate modifications on these amino acids. In particular, the presence of major $\mathbf{y}_{10}$ (*m/z* 1,100.8), $\mathbf{y}_{12}$ (*m/z* 1,312.9), and $\mathbf{y}_{14}$ (*m/z* 1,524.9) ions and minor $\mathbf{y}_{10}$-18 (*m/z* 1,081.6), $\mathbf{y}_{12}$-18 (*m/z* 1,295.4) ions argue strongly that these $\mathbf{y}_{10}$-18 and $\mathbf{y}_{12}$-18 ions are formed by the loss of water and not the β-elimination of phosphoric acid from ^{742}Ser. Due to the facile β-elimination of phosphoric acid, prominent $\mathbf{y}_{10}$-98 and $\mathbf{y}_{12}$-98 species would be observed if ^{742}Ser was the phosphorylated residue. The presence of $\mathbf{y}_{15}$-98 (*m/z* 1,593.0), $\mathbf{y}_{16}$-80 (*m/z* 1,724.6), and $\mathbf{y}_{16}$-98 (*m/z* 1,706.9) confirmed that the ^{737}Ser residue was modified with phosphate. The presence of ions corresponding to $\mathbf{b}_3$-98 (*m/z* 339.6) and $\mathbf{b}_7$-80 (*m/z* 782.1) and $\mathbf{b}_7$-98 (*m/z* 764.2) also indicated that ^{737}Ser was the modified residue.

Table 1 shows the peptides assigned to the RP/HPLC fractions following reflectron and linear mode MALDI/MS, and MALDI/MS with PSD analysis of the recombinant NBD1-R domain of CFTR. Masses corresponding to phosphopeptides containing the following CFTR PKA consensus sequence sites were bound to the NTA Sepharose and identified in the indicated RP/HPLC fractions (Table 1): 422, fraction 30; 660, fraction 12; 700, fractions 16 and 17; 712, fraction 8; 737, fractions 19 and 20; 768, fractions 10 and 17; 795, fraction 22; and 813, fraction 20. In addition, a mass consistent with a ^{753}Ser-containing tryptic phosphopeptide was observed in fraction 11. The phosphorylation of ^{422}Ser, ^{660}Ser,

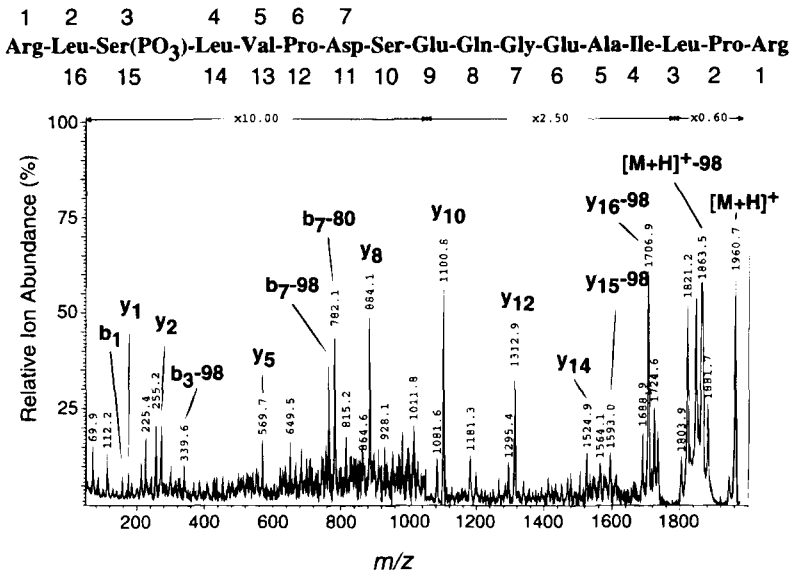

Fig. 4. PSD spectrum of the phosphorylated ^{735}Arg-Arg^{751} peptide from NBD1-R. The phosphopeptide of mass 1,961.2 from peak 19 (see Fig. 2A) was subjected to MALDI-PSD analysis. The N- (**b** ions) and C- (**y** ions) terminal sequence ions are indicated.

^{700}Ser, ^{737}Ser, ^{768}Ser, ^{795}Ser, and ^{813}Ser was confirmed by PSD analysis. The phosphorylation of ^{712}Ser and initially ^{753}Ser (see below) was inferred from the observed mass, metastable fragment ion formation, and PKA consensus phosphorylation sequence. Thirteen phosphopeptides bound to the IDA Sepharose and 12 to the NTA Sepharose. The difference in the number of phosphopeptides was from an incomplete or missed C-terminal tryptic cleavage of the ^{768}Ser-containing tryptic phosphopeptide, which, in this experiment, bound to the IDA Sepharose and not the NTA Sepharose. Masses inconsistent with tryptic peptides from NBD1-R were observed in fractions 7 and 29 and were not analyzed further.

The nonphosphorylated NBD1-R peptides that bound to the IDA Sepharose (Table 1) were categorized into the following groups: fractions 1, 11–16, 18–20, 23, and 24 contained 10 peptides from NBD1-R that have residues that result in nonspecific binding to IDA Sepharose (Glu, Asp, Lys, Tyr, Phe, Ala) (Muszynska et al., 1992); fractions 2–9 were comprised of poly-His-containing tryptic peptides from the His_6-tag sequence (Fig. 1); and fractions 2–4 and 24–28 were comprised of poly-Asp-containing tryptic peptides from the His_6-tag sequence (Fig. 1). The three nonphosphorylated peptides that bound to NTA Sepharose were seen in fractions 11, 13, 15, 16, and 19, whereas fractions 2 and 24–28 were comprised of poly-Asp-containing tryptic peptides from the His_6-tag sequence (Fig. 1). Thus, the NTA Sepharose is remarkably more specific for phosphopeptides than the IDA Sepharose.

Phosphopeptides from full-length CFTR, following in vitro phosphorylation and in-gel digestion, were purified by IMAC using the procedure described and NTA Sepharose. Using MALDI/MS analysis of the RP/HPLC-separated peptides, the same phosphopeptides were found as described for NBD1-R (Table 2) residues except for the ^{422}Ser-containing phosphopeptide. The phosphorylation of CFTR serines ^{660}Ser, ^{737}Ser, and ^{795}Ser was also confirmed by PSD analysis (data not shown). The peptide sequences and sites of phosphorylation of the ^{700}Ser-, ^{712}Ser-, ^{768}Ser-, and ^{813}Ser-containing peptides from CFTR were inferred from peptide mass, metastable fragment ion formation, C_{18} RP/HPLC elution position- and PKA consensus sequences.

Because the phosphorylation of ^{753}Ser has not been described for full-length CFTR, MALDI/CID was performed on the ^{12}C parent $[M+H]^+$ ion at *m/z* 1,422.6. The MALDI/CID spectrum provided more structural detail than the PSD spectrum, as shown in Figure 5. The immonium ions at *m/z* 60.1 (Ser), *m/z* 70.1 (Arg/Pro), *m/z* 86.1 (Ile/Leu), and *m/z* 101.1 (Lys/Gln) revealed partial amino acid composition of the phosphopeptide. The primary sequence ion series (**y** and **b** ions) were again used to determine site of phosphorylation and sequence identity, as with the PSD analysis. Gas-phase β-elimination of the phosphoric acid moiety again was the preferred metastable fragmentation (as was observed in the PSD spectra of the other phosphopeptides). The observed **y** ions [$\mathbf{y}_1$ (*m/z* 175.3), $\mathbf{y}_2$ (*m/z* 246.1), $\mathbf{y}_4$ (*m/z* 487.4), $\mathbf{y}_7$ (*m/z* 742.4), $\mathbf{y}_8$ (*m/z* 843.4), $\mathbf{y}_9$ (*m/z* 930.6), $\mathbf{y}_{10}$ (*m/z* 1,043.4), and $\mathbf{y}_{11}$ (*m/z* 1,143.1)] were used to confirm both the peptide sequence and assignment of nonphosphorylated residues. These results demonstrated that neither ^{756}Ser, ^{757}Thr, nor ^{760}Thr, the other sites that could potentially be phosphorylated, were modified. The presence of the $\mathbf{b}_1$ (*m/z* 114.5), $\mathbf{b}_2$-80 (*m/z* 201.0), and a prominent $\mathbf{b}_3$-98 (*m/z* 282.3) ion series provided additional evidence that the ^{753}Ser residue was the only phosphorylated residue. This is the first demonstration of phosphorylation at ^{753}Ser in a nonmutated full-length CFTR molecule.

Discussion

This study was undertaken to enable the analysis of CFTR phosphorylation using mass spectrometry because previous studies to identify the phosphorylation sites on CFTR have given differing results and have not defined the ensemble(s) of sites that regulate CFTR (Cheng et al., 1991; Picciotto et al., 1992; Chang et al., 1993; Seibert et al., 1995). To establish the method, phosphopeptides were isolated from the NBD1-R domain protein of CFTR using a novel IMAC approach method. Fe^{3+}-loaded NTA Sepharose was much more specific for phosphopeptides than either Fe^{3+}-loaded IDA Sepharose or IDA POROS™ resin. The phosphorylated sites identified using each matrix were identical. However, the number of nonphosphorylated peptides from the His_6-tagged

Table 1. *Tryptic peptides from NBD1-R identified by MALDI/MS after IMAC*

Fraction number	IDA Sepharose	NTA Sepharose
1	^{689}QTGEFGEKR^{697}	
2	DLYDDDDKDR	DLYDDDDKDR
	GSHHHHHHGMASMTGGQQMGR[a]	
3	DLYDDDDKDR	
4	DLYDDDDKDR	
	GSHHHHHHGMASMTGGQQMGR[a]	
5	GSHHHHHHGMASMTGGQQMGR[a]	
6	GSHHHHHHGMASMTGGQQMGR[a]	
8	^{710}KFSpIVQK^{716}[,b]	^{710}KFSpIVQK^{716}
	GSHHHHHHGMASMTGGQQMGR[a]	
9	GSHHHHHHGMASMTGGQQMGR	
10	^{765}RRQSpVLNMTHSVNQGQNIHR^{785}[,a]	^{765}RRQSpVLNMTHSVNQGQNIHR^{785}[,a]
	^{765}RRQSpVLNMTHSVNQGQNIHRK^{786}[,a]	^{765}RRQSpVLNMTHSVNQGQNIHRK^{786}[,a]
11	^{717}TPLQMNGIEEDSDEPLER^{734}[,a]	^{717}TPLQMNGIEEDSDEPLER^{734}[,a]
	^{752}ISpVISTGPTLQAR^{764}	^{752}ISpVISTGPTLQAR^{764}
	^{601}ILVTSKMEHLK^{611}	
12	^{658}RNSpILTETLHR^{668}	^{658}RNSpILTETLHR^{668}
	^{421}TSNGDDSLFFSNFSLLGTPVLK^{442}	
	^{448}IERGQLLAVAGSTGAGK^{464}	
13	^{443}DINFKIER^{450}	^{717}TPLQMNGIEEDSDEPLER^{734}
	^{421}TSNGDDSLFFSNFSLLGTPVLK^{442}	
	^{448}IERGQLLAVAGSTGAGK^{464}	
14	^{717}TPLQMNGIEEDSDEPLER^{734}	
	^{443}DINFKIER^{450}	
15	^{717}TPLQMNGIEEDSDEPLER^{734}	^{717}TPLQMNGIEEDSDEPLER^{734}
16	^{766}RQSpVLNLMTHSVNQGQNIHRK^{786}	
	^{599}TRILVTSKMEHLKK^{612}[,a]	
	^{697}RKNSpILNPINSIR^{709}	^{697}RKNSpILNPINSIR^{709}
	^{717}TPLQMNGIEEDSDEPLER^{734}	^{717}TPLQMNGIEEDSDEPLER^{734}
17	^{766}RQSpVLNLMTHSVNQGQNIHRK^{786}	
	^{766}RQSpVLNLMTHSVNQGQNIHR^{785}	^{766}RQSpVLNLMTHSVNQGQNIHR^{785}
	^{698}KNSpILNPINSIR^{709}	^{698}KNSpILNPINSIR^{709}
	^{697}RKNSpILNPINSIR^{709}	^{697}RKNSpILNPINSIR^{709}
18	^{537}DNIVLGEGGITLSGGQR^{553}	
19	^{533}FAEKDNIVLGEGGITLSGGQR^{553}	^{533}FAEKDNIVLGEGGITLSGGQR^{553}
	^{735}RLSpLVPDSEQGEAILPR^{751}	^{735}RLSpLVPDSEQGEAILPR^{751}
20	^{599}TRILVTSKMEHLKK^{612}	
	^{533}FAEKDNIVLGEGGITLSGGQR^{553}	
	^{811}RLSpQETGLEISEEINEEDLK^{830}	^{811}RLSpQETGLEISEEINEEDLK^{830}
	^{735}RLSpLVPDSEQGEAILPR^{751}	^{735}RLSpLVPDSEQGEAILPR^{751}
21	^{811}RLSpQETGLEISEEINEEDLK^{830}	
22	^{793}KVSpLAPQANLTELDIYSR^{810}	^{793}KVSpLAPQANLTELDIYSR^{810}
	^{811}RLSpQETGLEISEEINEEDLK^{830}	
23	^{793}KVSpLAPQANLTELDIYSR^{810}	
	^{504}ENIIFQVSYDEYR^{516}	
24	DLYDDDDKDRWGSGFGELFEK[c]	DLYDDDDKDRWGSGFGELFEK[c]
	^{504}ENIIFQVSYDEYR^{516}	^{504}ENIIFQVSYDEYR^{516}
25	DLYDDDDKDRWGSGFGELFEK[c]	DLYDDDDKDRWGSGFGELFEK[c]
26	WGSGFGELFEK	DLYDDDDKDRWGSGFGELFEK[c]
27	DLYDDDDKDRWGSGFGELFEK	DLYDDDDKDRWGSGFGELFEK
28	DLYDDDDKDRWGSGFGELFEK	DLYDDDDKDRWGSGFGELFEK
30	^{420}KTSpNGDDSLFFSNFSLLGTPVLK^{442}	^{420}KTSpNGDDSLFFSNFSLLGTPVLK^{442}
31	^{420}KTSpNGDDSLFFSNFSLLGTPVLK^{442}	

[a]Peptides that contained 1–3 oxidized methionines.
[b]Sp, phosphorylated serine residue.
[c]These peptides contained an unknown modification causing a 14-Da or 21-Da increment.

NBD1-R protein that bound to NTA Sepharose (5) was significantly less than the number bound to IDA Sepharose (15). The nonphosphorylated acidic peptides from the NBD1-R domain that bound to the NTA Sepharose all contained a minimum of three Glu or Asp residues, but none contained Glu residues alone as the acidic amino acid, as was observed for IDA Sepharose chroma-

Table 2. *Tryptic peptides from CFTR identified by MALDI/MS experiments after in-gel digestion and IMAC using NTA Sepharose*

Fraction number	Mass observed	Mass calculated	Phosphopeptide
1	929.4	929.5	^{710}KFSpIVQK^{716}
2	2,584.0	2,584.3	^{765}RRQSpVLNMTHSVNQGQNIHR^{785}[a]
3	1,422.0[b]	1,422.7	^{752}ISpVISTGPTLQAR^{764}
5	1,419.7	1,419.7	^{658}RNSpILTETLHR^{668}
8	1,448.7	1,448.8	^{698}KNSpILNPINSIR^{709}
9	1,961.2	1,960.0	^{735}RLSpLVPDSEQGEAILPR^{751}
10	2,099.8	2,098.1	^{793}KVSpLAPQANLTELDIYSR^{810}
	2,413.2	2,412.1	^{811}RLSpQETGLEISEEINEEDLK^{830}

[a]Peptide contained oxidized methionine.
[b]Linear mass.

tography. Thus, there are apparently differences between Fe^{3+}-loaded NTA and IDA Sepharoses in their affinity for the different acidic residues, with Glu residues being more tightly bound to the IDA Sepharose. Of significance for analyzing recombinant proteins with His-tags, the IDA, but not the NTA, Sepharose bound to the basic poly-His-containing tryptic peptide from the His_6 sequence. This interaction is due to the proposed binding of His residues with free IDA groups (Scanff et al., 1991), where Fe^{3+} ions have been removed from or did not bind to IDA molecules on the resin. This also concurs with the finding that NTA-modified resins have better stability than IDA-modified matrices when tested for ion binding strength (Hochuli et al., 1987).

It is noted that Al^{3+}-loaded IDA Sepharose has been reported to be more selective than Fe^{3+}-loaded IDA Sepharose for phosphopeptide binding (Olcott et al., 1994) and that acidic peptides were not co-purified with phosphopeptide(s). However, Al^{3+}-loaded NTA Sepharose was less specific than either Fe^{3+}-loaded IDA Sepharose or Fe^{3+}-loaded NTA Sepharose for NBD1-R domain phosphopeptide binding when tested (data not shown). It also should be noted that peptide desalting prior to IMAC was necessary because the efficiency of phosphopeptide binding appeared to decrease when nondesalted peptide mixtures were analyzed (data not shown). This may be due to the presence of adenosine triphosphate, urea, and other salts. ATP is known to bind to Fe^{3+}-loaded matrices (Muszynska et al., 1992) and, when present in large excess over phosphopeptide, may compete with and/or block phosphopeptide binding.

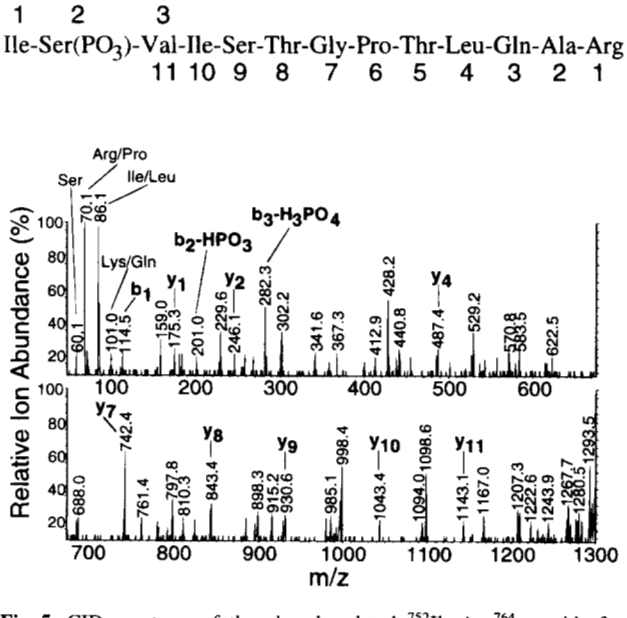

Fig. 5. CID spectrum of the phosphorylated ^{752}Ile-Arg^{764} peptide from CFTR. The phosphopeptide of mono-isotopic mass 1,422.6 was subjected to MALDI-CID analysis. The N- (**b** ions) and C- (**y** ions) terminal sequence ions are indicated. The matrix was 2,5-dihydroxybenzoic acid.

The specificity of NTA versus IDA matrices may be due to the following factors. NTA (a quadradentate metal-chelator) Sepharose and IDA (a tridentate metal-chelator) Sepharose bind Ni^{2+} ions (with a coordination number of six), while leaving free two and three coordination sites, respectively (Hochuli et al., 1987). NTA-modified resins have higher affinity for metal ions with coordination numbers of six and four, and greater stability than IDA-modified matrices (Hochuli et al., 1987). Because Fe^{3+} ions also have a coordination number of six, Fe^{3+}-loaded NTA Sepharose should be more stable than IDA Sepharose. Also, IDA Sepharose may lose the bound Fe^{3+} ions on sample loading because metal ions with a coordination number of six have less affinity for IDA. Furthermore, the fact that a third coordination site remains free in IDA Sepharose may contribute to the greater nonspecific interactions not seen with NTA Sepharose. However, Fe^{3+}-loaded NTA-modified resins, in contrast with Fe^{3+}-loaded IDA-modified resins (Andersson & Porath, 1986; Muszynska et al., 1986, 1992; Michel & Bennet, 1987; Flotow et al., 1990; Scanff et al., 1991; De Ancos & Avila, 1993), have not been reported as ligands for phosphopeptide or phosphoprotein analysis.

MALDI/MS analysis of NBD1-R phosphorylation following IMAC and RP-HPLC demonstrated that ^{422}Ser, ^{660}Ser, ^{700}Ser, ^{712}Ser, ^{737}Ser, ^{753}Ser, ^{768}Ser, ^{795}Ser, and ^{813}Ser were phosphorylated by PKA. An earlier study of NBD1-R phosphorylation using LC-ESI/MS demonstrated that the same sites were phosphorylated by PKA, except for the ^{753}Ser site (Townsend et al., 1996). The phosphorylation of ^{753}Ser was inferred from PKA consensus sequence matching after a mass consistent with a phosphorylated ^{752}Ile-Arg^{764} peptide was observed. This phosphorylation site had been found previously only in vivo with a mutated (10Ser-Ala) CFTR protein (Seibert et al., 1995). The identification of the ^{752}Ile-Arg^{764} sequence also shows that trypsin can cleave proteins at the

−2 position from a phosphorylated residue, in the sequence Arg/Lys-Xxx-Ser(PO_4), not reported to occur previously (Boyle et al., 1991). Trypsin cleaved all other phosphopeptides at the expected −3 position in the sequence Arg/Lys-Arg/Lys-Xxx-Ser(PO_4). This demonstrated that IMAC purification allows for the identification of phosphorylation sites not observed previously by mass spectrometry on non-IMAC purified samples.

The identification of phosphoserine-containing peptides by MALDI/MS in reflectron mode was facilitated by the formation of metastable fragments from the loss of HPO_3 and H_3PO_4 from the parent ion, with the metastable fragments from the loss of H_3PO_4 always being the more abundant ion formed. This fragmentation pattern is characteristic of phosphoserine and phosphothreonine residues, but not phosphotyrosine (Annan & Carr, 1996) or phosphohistidine residues (Medzihradszky et al., 1997) because the loss of H_3PO_4 is not observed. This is probably due to the fact that β-elimination of H_3PO_4 cannot occur and thus a loss of 98 Da will not be observed. The amount of metastable fragmentation appeared to be dependent on phosphopeptide sequence because the relative abundance of parent, parent-HPO_3, and parent-H_3PO_4 ions was not consistent. These fragments were formed post source, because they were not detected at precisely 80- and 98-Da increments, respectively, from the parent phosphopeptide ions. When the same phosphoserine-containing peptides were examined in linear mode, these fragments were not observed because both parent and daughter ions were detected at the same time. Therefore, reflectron and linear modes are complementary in the MALDI/MS analysis of phosphopeptides.

IMAC following in-gel digestion of large membrane proteins has been reported (De Jongh et al., 1993), but large amounts (>500 pmol) of purified protein were labeled with [^{32}P] prior to in-gel digestion. However, the sites of phosphorylation and the presence of phosphoserine was inferred from the relative amounts of PTH-serine to PTH-dehydroalanine when NH_2-terminal sequencing the C_{18} RP/HPLC-separated peptides. PTH-dehydroalanine is formed from phosphoserine during the PTH derivatization reaction. No data were presented for nonphosphorylated proteins co-purified using the IDA matrix.

IMAC-MALDI/MS of full-length CFTR, following in-gel digestion, identified eight phosphopeptides. By this novel approach, the CFTR sites that are phosphorylated by PKA in vitro were unambiguously identified. No phosphopeptides were detected in the control (non PKA-phosphorylated) CFTR sample and thus it was concluded that phosphorylated residues were absent, or below detectable levels, prior to PKA treatment. The phosphorylation of Ser residues 660, 700, 712, 737, 753, 768, 795, and 813 was demonstrated. Phosphorylation of ^{422}Ser, as observed in the PKA-phosphorylated NBD1-R domain, was not detected in full-length CFTR. The ^{422}Ser site may not be accessible to PKA due to differences in protein structure between CFTR and the expressed NBD1-R domain. This may be due to the presence of the His-tag sequence in the recombinant NBD1-R domain protein, which may influence protein folding and thus accessibility to PKA. It was shown conclusively that ^{753}Ser is phosphorylated by PKA in the full-length protein, supporting the hypothesis that modification at this site results in up to 25% of the channel activity (Seibert et al., 1995). The advantage of IMAC, employing Fe^{3+}-loaded NTA Sepharose, combined with MALDI/MS over site-specific mutagenesis and phosphopeptide mapping is clearly shown. The data presented here for CFTR gave identification of peptide sequence and site of phosphorylation following MALDI mass analysis and MALDI/PSD or MALDI/CID sequence analysis. IMAC using NTA Sepharose followed by MALDI/MS should have numerous applications for analysis of both in vitro and in vivo phosphorylation because subpicomole amounts of phosphopeptide can be sequenced by MALDI/PSD (Annan & Carr, 1996).

Materials and methods

Materials

Ni-NTA Sepharose CL-6B was purchased from Qiagen (Chatsworth, California) (supplied as Ni-NTA Agarose), metal-chelating IDA Sepharose was from Pharmacia Biotech (Piscataway, New Jersey), IDA POROS™ was from PerSeptive Biosystems (Cambridge, Massachusetts), and sequencing grade trifluoroacetic acid was from Pierce (Rockford, Illinois). cAMP-dependent protein kinase A (catalytic subunit) and sequencing grade trypsin were from Promega (Madison, Wisconsin). All other reagents were from Fisher or Sigma (St. Louis, Missouri).

Purification of the NBD1-R domain of CFTR

His_6-tagged NBD1-R domain (amino acids 404–830 of human CFTR) was expressed and purified from *Escherichia coli* as described previously (Townsend et al., 1996). The protein (1.5 mg/mL) was stored at −20 °C in 8 M urea, 100 mM sodium phosphate, 10 mM Tris-HCl, pH 5.0.

Phosphorylation and trypsin digestion of NBD1-R

Phosphorylation of NBD1-R (1 nmol) was performed in a solution containing 10 mM magnesium sulfate, 10 mM ATP (magnesium salt), and 0.1 M *N*-ethyl morpholine in a final volume of 140 μL. PKA (63 U/μL) was added (2.5 μL) and the mixture was incubated at 37 °C for 90 min. Freshly prepared trypsin (1 μg/μL) was added (2.5 μL in 0.1 M *N*-ethyl morpholine) and the reaction mixture was further incubated at 37 °C for 90 min. The reaction was terminated by the addition of 3 μL of trifluoroacetic acid.

Peptide desalting by RP/HPLC

Chromatography was performed with a 1.0 × 150 mm Reliasil C_{18} column (5 μm, 300-Å packing) using a microbore HPLC system (both from Michrom BioResources, Inc., Auburn, California). The flow rate was 50 μL/min and peptides were detected by post-column UV absorbance (210 nm). Solvent A was 2% acetonitrile, 0.1% trifluoroacetic acid in water and solvent B was 98% acetonitrile, 0.08% trifluoroacetic acid in water. The column was equilibrated in 2% solvent B. Samples were loaded and the column was eluted isocratically with 2% solvent B to remove unbound material. When the absorbance returned to baseline, the eluant was stepped to 80% solvent B over 2 min and the eluted peak(s) collected in a 0.65-mL microcentrifuge tube. The samples were dried to approximately 20 μL using a Speed Vac, and 80 μL of 1 M acetic acid was added to the peptide mixture.

CFTR baculovirus construct generation

Site-directed mutagenesis (Kunkel, 1985) was used to introduce unique *Not* I restriction endonuclease sites flanking the CFTR cDNA coding sequence at positions 89 and 4594. An octapeptide

epitope "flag" (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was inserted into the carboxy-terminus of CFTR replacing amino acids 1472–1479 of the wild-type protein. The construct was excised from pBluescript using *Not* I and subcloned into the baculovirus transfer vector pVL1392 (Invitrogen, Carlsbad, California). Plaque-pure recombinant baculovirus was generated according to established procedures (Webb & Summers, 1990).

Production and purification of recombinant CFTR

Hi5 insect cells in T150 tissue culture flasks were infected with the baculovirus construct at a multiplicity of infection of 1–5. Three days post infection, the cells were dislodged, pelleted at 1,500 × *g* for 5 min, and resuspended in lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1% Triton X-100, 0.8% CHAPS, 1 mM Pefabloc, 1 μg/mL leupeptin, and 5 μg/mL aprotinin) containing 0.5 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mg/mL para-nitrophenyl phosphate. Three milliliters of lysis buffer were used per T-150 flask. The insoluble material was pelleted at 40,000 × *g* for 30 min and the supernatant was incubated for 1 h at 4 °C with 100 μL (packed bed volume) of anti-flag M2-agarose beads (Eastman Kodak Co., Scientific Imaging Systems, Rochester, New York) per 3 mL lysate. The beads were then washed with four changes of lysis buffer (250 volumes). Bound proteins were eluted from the beads using 0.1 M glycine, 0.1% Triton X-100, pH 3.0, and adjusted to pH 7.4 with Tris base. The protein was stored at −80 °C. This immunoaffinity-purified epitope-tagged CFTR is termed full-length CFTR.

Phosphorylation and SDS-PAGE of CFTR

Aliquots (300 μL) of 100 mM magnesium sulfate and 100 mM ATP were added to separate solutions (3 mL) of purified CFTR. The solutions were vortexed and an aliquot (15 μL) of PKA (63 U/μL) was added to one. The second solution was the nonphosphorylated control. The reactions were placed at 37 °C for 3 h. Protein was precipitated using chloroform/methanol (Wessel & Flügge, 1984). Pellets were dissolved in SDS-PAGE solubilization buffer [6 M urea, 2% SDS, 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, and 0.02% (w/v) bromophenol blue]. Samples were allowed to stand in solubilization buffer for at least 30 min at room temperature before electrophoresis.

Samples were electrophoresed through an 8% gel at constant current (10 mA) using a Mini-Protean II gel electrophoresis apparatus (BioRad, Hercules, California) and a discontinuous buffer system (Laemmli, 1970). Gels were stained in 0.1% Coomassie blue G-250 in 50% methanol and 1% acetic acid in water for 30 min.

In-gel digestion of CFTR

The Coomassie blue-stained CFTR band(s) were excised from the gel and diced into small pieces (~2 × 2 mm) and placed into 0.65-mL siliconized microcentrifuge tubes. The proteins were destained and digested using modifications to published procedures (Rosenfeld et al., 1992; Hellman et al., 1995; Jeno et al., 1995). Coomassie blue was removed by three washes (20 min each) using 25 mM ammonium bicarbonate and 50% acetonitrile in water. A volume sufficient to cover the gel pieces (~275 μL) plus an additional 100 μL was used for each wash. The gel was dried in a Speed Vac and freshly prepared sequencing grade trypsin (0.025 μg/μL in 25 mM NH_4HCO_3) was added until the gel was fully rehydrated. The reaction mixture was placed at 37 °C for 16–20 h. The reaction mixture was centrifuged at 14,000 rpm for 30 s and the digest solution was transferred into a clean 0.65-mL siliconized microcentrifuge tube. The remaining tryptic peptides were extracted from the gel by two washes (20 min each) using 80% acetonitrile and 2% trifluoroacetic acid in water. All of the washes were pooled and dried in a Speed Vac to a total volume of approximately 50 μL and an equal volume of 1 M acetic acid was added prior to subsequent processing.

Purification of phosphopeptides using IMAC

The procedure was a modification of the method of Nuwaysir and Stults (1993). Columns were prepared by pouring each resin (final packed bed volume was 0.5 mL) into separate 10-mL Polyprep columns (BioRad, Hercules, California). The resins were packed using gravity flow. The columns were washed with 2 mL of water followed by 2 mL of 0.1 M EDTA to remove any bound divalent metal ions. The columns were then washed sequentially with water (2 mL), 0.1 M acetic acid (2 mL), and 0.1 M ferric chloride in 0.1 M acetic acid (2 mL) to load the columns with Fe^{3+} ions. Two milliliters of 0.1 M acetic acid were then used to elute any unbound Fe^{3+} ions.

For the resin specificity experiments, 300-pmol aliquots of the same phosphorylated, trypsinized, and desalted NBD1-R sample were loaded (~ 30 μL) in acetic acid (pH < 3.5) onto the columns. For analysis of CFTR phosphopeptides, the in-gel digested samples (~100 μL) were loaded onto NTA Sepharose columns following peptide recovery. The columns were washed with four column volumes of 0.1 M acetic acid followed by sequential washes with two column volumes of water and 0.1% ammonium acetate (adjusted to pH 8.0 with ammonium hydroxide). Bound peptides were eluted with four column volumes of 0.1% ammonium acetate adjusted to pH 9.5 with ammonium hydroxide. The eluted fraction was dried in a Speed Vac and dissolved in 50 μL of 5% acetonitrile in water, containing 1% trifluoroacetic acid. All columns were pre-cycled with all eluants before sample loading. Alternatively, the columns can be regenerated by washing with water (2 mL) followed by 0.1 M EDTA (2 mL) (to remove any bound metal ions) and reloaded with Fe^{3+} ions using the procedure outlined above.

Peptide separation by RP/HPLC

Peptides eluted at pH 9.5 from the iron-chelation columns were separated and detected using the C_{18} column and microbore system described. The column flow rate was 50 μL/min. The sample was injected onto a column that had been equilibrated in 2% solvent B. The column was eluted isocratically with 2% solvent B for 10 min followed by a linear gradient to 50% solvent B over 50 min. The detected peptides were collected into 0.65-mL microcentrifuge tubes, dried to ~1 μL, and 5 μL of 50% acetonitrile in water, containing 5% trifluoroacetic acid, were added.

MALDI/MS analysis of the RP/HPLC-fractionated IMAC-bound peptides

Molecular masses of the tryptic peptides were determined using a TofSpec SE mass spectrometer from Micromass (Manchester, UK) equipped with a reflectron and a nitrogen laser (337 nm). A 1-μL

aliquot of each fraction was mixed with 1 μL of α-cyano-4-hydroxycinnamic acid (Hewlett-Packard, Wilmington, Delaware) and 1 μL was spotted onto a stainless steel target and allowed to air dry. All data were collected as positive-ion spectra. MALDI spectra were calibrated externally by use of a standard peptide mixture. In PSD analysis, performed using the described mass spectrometer, the fragments generated by metastable decay of the molecular species of interest were focused by stepping the voltage of the reflectron. The spectra recorded at each step were combined after calibration to generate a composite PSD spectrum.

MALDI-CID analysis was performed, in positive-ion mode, using an AutoSpec 5000 orthogonal acceleration (OA)-time-of-flight mass spectrometer from Micromass. MS-1 is a AutoSpec 5000 double-focusing magnetic sector mass spectrometer equipped with a MALDI source and a nitrogen laser (337 nm). MS-1 was tuned manually to transmit the ^{12}C monoisotopic $[M+H]^+$ precursor mass and CID fragmentation was performed as described (Medzihradszky et al., 1996). The sample (1 μL) was mixed with 2,5 dihydroxybenzoic acid (1 μL of a saturated solution in acetone with 2% water and 5% trifluoroacetic acid) and spotted onto a stainless steel target and allowed to air dry.

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