Disulfide Bond Assignments of Secreted Frizzled-related Protein-1 
Provide Insights about Frizzled Homology and Netrin Modules*

Secreted Frizzled-related protein-1 (sFRP-1), a soluble protein that binds to Wnts and modulates Wnt signaling, contains an N-terminal domain homologous to the putative Wnt-binding site of Frizzled (Fz) domain and a C-terminal heparin-binding domain with weak homology to netrin. Both domains are cysteine-rich, having 10 and 6 cysteines in the Fz and heparin-binding domains, respectively. In this study, the disulfide linkages of recombinant sFRP-1 were determined. Numbering sFRP-1 cysteines sequentially from the N terminus, the five disulfide linkages in the Fz domain are 1–5, 2–4, 3–8, 6–10, and 7–9, consistent with the disulfide pattern determined for homologous domains of several other proteins. The disulfide linkages of the heparin-binding domain are 11–14, 12–15, and 13–16. This latter set of assignments provides experimental verification of one of the disulfide patterns proposed for netrin (NTR) modules and thereby supports the prediction that the C-terminal heparin-binding domain of sFRP-1 is an NTR-type domain. Interestingly, two subsets of sFRPs appear to have alternate disulfide linkage patterns compared with sFRP-1, one of which involves the loss of a disulfide due to deletion of a single cysteine from the NTR module, whereas the remaining cysteine may pair with a new cysteine introduced in the Fz domain of the protein. Analysis of glycosylation sites showed that sFRP-1 contains a relatively large carbohydrate moiety on Asn172 (~2.8 kDa), whereas Asn262, the second potential N-linked glycosylation site, is not modified. No O-linked carbohydrate groups were detected. There was evidence of heterogeneous proteolytic processing at both the N and C termini of the recombinant protein. The predominant N terminus was Ser31, although minor amounts of the protein with Asp41 and Phe39 as the N termini were observed. The major C-terminal processing event was removal of the terminal amino acid (Lys313) with only a trace amount of unprocessed protein detected.

Wnt signaling has been implicated in the specification of cell fate, polarity and proliferation, tissue patterning, and the onset of neoplasia (reviewed in Refs. 1 and 2). Signaling is initiated by the secreted Wnt proteins, which react with proteins on the cell surface to form a receptor complex consisting of a seven-pass transmembrane molecule of the Frizzled (Fz) family (3) and either LRP5 or LRP6/Arrow (4–6), members of the low density lipoprotein receptor-related family (7, 8). In the absence of Wnt receptor activation, the modular protein Axin provides a scaffold for the binding of glycogen synthesis kinase 3β (GSK-3β), adenomatous polyposis coli (APC) protein, and β-catenin (9–13). This facilitates the phosphorylation of β-catenin by GSK-3β and subsequent rapid degradation of β-catenin by a ubiquitin-dependent process (14, 15). In response to Wnt binding, the Axin-GSK-3β-APC-β-catenin complex is disrupted by a process that involves the cytoplasmic proteins Dishevelled and Frat (16, 17–20), dephosphorylation of Axin (22, 23), and recruitment of Axin to LRP5 associated with Axin destabilization (24). As a result, the phosphorylation and degradation of cytosolic β-catenin are inhibited, leading to its interaction with DNA-binding proteins of the T-cell factor/lymphoid enhancer-binding factor family and accumulation in the nucleus where these complexes activate expression of target genes (25–30). Mutations in APC, β-catenin, and Axin that increase the steady state level of soluble β-catenin create conditions tantamount to a constitutively active canonical Wnt pathway and have been observed in many human cancers (reviewed in Ref. 2).

The Wnt-binding site in Fz proteins consists of ~120 amino acid residues and has been designated the Fz cysteine-rich domain (CRD) because it contains 10 cysteines that are present in all members of the Fz family (3, 31). Several other proteins possessing a Fz CRD have been identified, including tyrosine kinases (32, 33), carboxypeptidase Z (34), and an isoform of collagen XVIII (35). In addition, a set of secreted Fz-related proteins (sFRPs) have been described that are ~300 amino acids in length and contain an N-terminal Fz CRD that is typically ~30–50% identical to the CRDs of Fzs (36–46). These proteins bind Wnts and regulate their activity in a variety of assays. Although the Wnt binding of sFRPs is generally believed to be mediated by the Fz CRD, interaction between Wingless (Drosophila ortholog of mammalian Wnt1) and a sFRP-1 mutant lacking the CRD imply that other mechanisms of direct or indirect interaction also exist (47).

The C-terminal heparin-binding portion of sFRPs bears weak homology with netrins (36, 37), proteins involved in axonal guidance (48). Originally, this potential relationship was based on the presence of clusters of positively charged residues...

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The abbreviations used are: Fz, Frizzled; LRP, low density lipoprotein receptor-related protein; sFRP, secreted Frizzled related protein; CRD, cysteine-rich domain; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; TCEP, tris(2-carboxyethyl)phosphine; MDCK, Madin-Darby canine kidney; CNBr, cyanogen bromide; NTR, netrin; PCOLCE, procollagen C-proteinase enhancer protein; WFIKKN, WAP, Fas, Ig, Ks, and NTR protein; TIMP, tissue inhibitors of metalloproteinases; Szl, Sizzled; RP-HPLC, reversed phase-high performance liquid chromatography; GSK-3β, glycogen synthesis kinase 3β; Tricine, N-tris(hydroxymethyl)methylglycine.
and a few other conserved amino acids distributed over a span of ~50 amino acids in FzR2/sFRP-3 (36). More recently, Bányaí and Pathy (49) identified a netrin (NTR) module in the C-terminal domains of netrins, sFRPs, type I procollagen C-proteinase enhancer proteins (PCOLCEs), complement proteins C3, C4, and C5, and in the N-terminal domains of tissue inhibitors of metalloproteinases (TIMPs). This homology was based on related patterns of six conserved cysteines, several conserved segments of hydrophobic residues, and a correlation between predicted and known secondary structure in some of the proteins having the domain. However, experimentally determined disulfide bond assignments for the cysteine residues were only available for TIMPs and complement protein C3, the latter being a variant in the group that contains only four of the conserved cysteines. Thus, the validity of the proposed NTR module would be reinforced if the disulfide structure of another protein containing the putative domain conformed to the predicted scheme.

In this study, we characterized the post-translational processing of sFRP-1. The linkages of the eight disulfide bonds and the site of N-linked glycosylation in sFRP-1 were determined using MALDI-MS and N-terminal sequencing of purified peptides. The data show that sFRP-1 has two distinct domains with 10 and 6 cysteines in the N- and C-terminal domains, respectively. The N-terminal domain has a pattern of disulfide linkages identical to that of the Fz CRD recently defined in rat tyrosine kinase Ror-1, mouse sFRP-3, and mouse Fz8 (50, 51). The assignment of disulfides in the C-terminal domain experimentally validates the primary disulfide pattern predicted for NTR modules (49). In addition, these results provide the first complete experimental assignment of disulfide linkages in an sFRP recombinant protein containing both a CRD and an NTR domain in tandem. An interesting aspect of this assignment is that two other subsets of sFRPs are likely to have different disulfide linkages compared with sFRP-1, suggesting that shuffling of several disulfide bonds may have occurred during evolution of this protein family.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trypsin (sequencing grade) was purchased from Promega (Madison, WI). Subtilisin was obtained from Roche Molecular Biochemicals. Tris-(2-carboxyethyl)-phosphine (TCEP) was obtained from Pierce. Cyanogen bromide (CNBr) was obtained from Aldrich. Reagents for PAGE were obtained from Bio-Rad. All other reagents were either high performance liquid chromatography (HPLC) grade or the highest quality analytical reagent grades available.

**Purification of sFRP-1**—Recombinant human sFRP-1 was purified from MDCK cell culture supernatants as described previously (47).

**CNBr Fragmentation**—CNBr was used for initial fragmentation of sFRP-1 for disulfide assignments because the intact unreduced protein was unusually resistant to cleavage by all proteases tested. Acetic acid (5% final concentration) was added to purified sFRP-1 (1.8 mg/2 ml), and the protein was desalted on an Econo-Pac10DG desalting column (Bio-Rad) using 5% acetic acid to elute the protein. Fractions containing protein were pooled, lyophilized, and reconstituted in 88% formic acid (Bio-Rad) using 5% acetic acid to elute the protein. Fractions containing protein were pooled, lyophilized, and reconstituted in 88% formic acid (Bio-Rad) using 5% acetic acid to elute the protein. Fractions containing protein were pooled, lyophilized, and reconstituted in 88% formic acid (Bio-Rad) using 5% acetic acid to elute the protein. Fractions containing protein were pooled, lyophilized, and reconstituted in 88% formic acid (Bio-Rad) using 5% acetic acid to elute the protein.

**Partial Reduction with TCEP and Alkylation**—TCEP partial reduction of peptide complexes containing multiple disulfides was performed as described previously (52). The purified peptide complex (160 pmol/50 μl) was mixed with an equal volume of 20 mM TCEP in 50 mM citrate, pH 3.2, and incubated for 3 min at 22 °C. Alkylation of peptides was performed by adding the TCEP-reduced peptide solution into an equal volume of 1 M iodoacetamide in 200 mM HEPES, 2 mM EDTA, pH 8.0, followed by incubation at 37 °C for 30 min. The reaction was stopped by adding 1.3% trifluoroacetic acid (final concentration).

**Site-directed Mutagenesis at Asn172 and Asn262**—Single amino acid substitutions were introduced with sFRP-1/pcDNA3.1 (47) as template and the QuickChange XL Site-directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions.

**Protein Purification and Characterization of sFRP-1**—Recombinant sFRP-1 was purified from MDCK cell culture supernatant by heparin-Sepharose affinity chromatography. The purified protein migrated on SDS-PAGE with an apparent mass of ~35 kDa (Fig. 1A). MALDI-MS analysis of purified sFRP-1 showed a single broad peak with an average mass (MH+) of 35,452 Da. N-terminal sequence analysis of sFRP-1 indicated that the majority of the polypeptide chains began with Ser31, whereas ~10% and ~7% of the sample began with Asp41 and Phe312, respectively (Fig. 2). MALDI-MS analysis of cyanogen bromide peptides revealed the presence of C-terminal variants of sFRP-1 to be Phe312, although trace amounts of the protein with C termini at Gln309, Ser310, Phe308, Val311, and Lys313 were observed (data not shown). Because the calculated amino acid sequence mass of sFRP-1(Ser31-Phe312) is 32,394 Da, the difference between observed and calculated mass suggested the molecule was glycosylated where the major species had ~30,000 Da of carbohydrate mass. The broad MS peak shape was consistent with
heterogeneity of the putative carbohydrate moiety and heterogeneous proteolytic processing of the N and C termini described above.

CNBr Fragmentation—Initial fragmentation of sFRP-1 utilized CNBr to cleave peptides on the C-terminal side of methionines, which resulted in conversion of these residues to a mixture of homoserine and homoserine lactone. Masses corresponding to both methionine derivatives were observed for most peptides. For simplicity, only masses corresponding to the predominant homoserine lactone form are reported (residue mass/charge = 83.04 Da), and these residues are indicated as Met when peptide sequences are described. The CNBr fragments were separated by HPLC gel filtration, and major peaks/pools were designated by C1 to C6 as shown in Fig. 3A. The protein bands observed in fractions C1, C2, and C3 on nonreducing gels shifted to lower molecular weight positions on reducing gels, which indicated these fractions contained disulfide linkages (Fig. 3, B and C). MALDI-MS of C1 showed a single broad peak with an average mass of 30,428.7 Da prior to reduction, whereas masses corresponding to Ser–Met, Ala–Met, and a weak signal for Lys–Phe were observed after reduction (Table I). Comparisons of SDS gel bands and masses of C1, C2, and C3 showed that Met had not been cleaved in C1 resulting in isolation of a single large unreduced complex containing all disulfide-linked peptides. This incompletely fragmented CNBr peptide was not directly identified in the MS analysis apparently due to a combination of its large size and the glycosylated moiety on this fragment that interfered with ionization of the peptide after reduction (see below). The C2 peptide complex contained the six cysteines from the heparin-binding (NTR) domain in three polypeptide chains: glycosylated Thr–Met, Lys–Met, and Lys–Phe, which confirmed the major C terminus of the protein was Phe. The C3 peptide complex contained the 10 cysteines from the Fz CRD domain in three polypeptide chains: Ser–Met, Ala–Met, and Leu–Met. The C4 to C6 peptide fractions did not contain any cysteine residues and were determined to be Gly–Met, Gln–Met, and Val–Met respectively. Peaks C1 to C3 were further analyzed as described below to determine the disulfide linkages of sFRP-1.

Analysis of the C-terminal Heparin-binding Domain—The total mass of the unreduced C2 peptide complex indicated a

FIG. 1. SDS-PAGE and MALDI-time-of-flight-mass analysis of purified sFRP-1. A, heparin affinity-purified recombinant sFRP-1 was separated on a 15% Tris-Tricine gel under nonreducing conditions and stained with Coomassie Blue. The positions of standard proteins in kDa are shown on the left. B, MALDI-time-of-flight-MS of purified sFRP-1. One broad singly charged species is observed with an average mass (MH) of 35,452 Da. The peaks at 44,614 and 22,308 Da are singly and doubly charged ions (M+2H) of protein A, respectively, which is an internal standard.

FIG. 2. Amino acid sequence of sFRP-1 summarizing experimentally determined disulfide linkages, N-glycosylation sites, and proteolysis processing. The predominant N- and C-terminal processing is indicated by solid arrows at Ser and Phe, respectively. Minor alternative heterogeneous N termini resulting from proteolytic processing at Asp and Phe are indicated by dashed arrows. Bold lines between cysteines indicate assigned disulfide bonds. The glycosylated N-linked site on Asn is indicated by a solid underline and the unmodified site at Asn is indicated by a dashed underline. The CNBr cleavage sites are indicated by m. The major sites of cleavage by subtilisin to produce peptides used to define disulfide linkages are indicated by open arrowheads.
disulfide-linked complex containing Thr<sup>169</sup>–Met<sup>210</sup>, Lys<sup>211</sup>–Met<sup>270</sup>, and Lys<sup>301</sup>–Phe<sup>312</sup>, plus an additional mass of 2812 Da that proved to be due to glycosylation. Because C2 contained three disulfide bonds, further cleavage with subtilisin (enzyme: substrate = 1:3 (w/w)) was used. Representative RP-HPLC chromatograms of the C2 subtilisin digest before and after reduction are shown in Fig. 4. All peak fractions in the nonreduced chromatogram and selected peaks in the reduced chromatogram were analyzed by MALDI-MS. Peptides that could not be unambiguously identified by mass analysis were subjected to Edman sequencing. Three major peaks observed in the nonreduced digest (C2-S1 to C2-S3, upper panel of Fig. 4) were observed to be disulfide-linked complexes. In addition, several new peaks appeared in the reduced subtilisin digest chromatogram that corresponded to cysteine-containing peptides released from disulfide linkages after reduction. The C2-S1 com-
Asn251, and Gly252, whereas the C2-S3 complex was the same in two polypeptide chains, Gly181
separation of 5 solvent B over 75 min; and 32 procedures
ZORBAX 300SB-C18 column as described under "Experimental Procedures" using the following gradient: 2% solvent B for 5 min; 2–32% solvent B over 35 min. Lower panel, separation of 5 µg of C2 subtilisin digest after reduction with TCEP using the same gradient. Major peaks that disappeared following reduction are indicated by C2-S1 to C2-S3. MALDI-MS analyses of these fractions are summarized in Table II.

complex had two cysteines in two polypeptide chains, Cys202–Ser204 and Lys109–Gln109, giving one direct disulfide assignment of Cys202–Cys205 (Table II). The C2-S2 complex had four cysteines in two polypeptide chains, Gly181–Lys183 and Leu249–Leu263, with heterogeneous cleavage at the C termini of Thr192, Lys256, Asn251, and Gly252, whereas the C2-S3 complex was the same as C2-S2 with heterogeneous cleavage only at Thr192. Because further attempts to cleave between adjacent cysteines in C2-S2 and C2-S3 were not successful, the C2-S3 complex was subjected to partial reduction using TCEP followed immediately by alkylation with iodoacetamide and subsequent separation by RP-HPLC. The results from MALDI-MS and Edman sequence analyses of the partially reduced and alkylated C2-S3 are summarized in Table III. Peptides C2-S3-R1 to C2-S3-R3 were identified as completely reduced and alkylated single polypeptides. C2-S3-R4 consisted of two disulfide-linked polypeptides, Gly181–Thr183–Lys185 and Leu249–Leu263, with one alkylated cysteine on each peptide. Edman sequencing of C2-S3-R4 showed that Cys188 and Cys257 were alkylated, indicating that Gly181–Thr183–Lys185 and Leu249–Leu263 were linked by the disulfide bond Cys185–Cys255. These data also indicated that the two reduced and alkylated residues, Cys188 and Cys257, in C2-S3-R4, represent the remaining disulfide bond in the original complex. Therefore, the complete disulfide bond assignments of the sFRP-1 C-terminal heparin-binding domain were determined to be Cys185–Cys255, Cys188–Cys257, and Cys202–Cys205.

Analysis of Disulfide Bonds in the N-terminal Fz CRD Domain—The C3 peptide complex resulting from CNBr fragmentation consisted of Ser31–Met75, Ala87–Met143, and Leu154–Met168, linked by five disulfide bonds. The C3 peptide complex was subjected to subtilisin digestion (E:S = 1:3 (w/w)) and RP-HPLC to further separate disulfide-linked complexes (Fig. 5). Several peaks observed in the nonreduced digest (C3-S1 to C3-S7) were not observed in the reduced digest, indicating the presence of disulfide-linked complexes. The C3-S1 to C3-S3 complexes had a total of two cysteines in two polypeptide chains, Cys104–Glu109 and Ala134–Met143 with heterogeneous cleavage at the C termini of Ala134 and Ser138, giving a direct disulfide assignment of Cys104–Cys139 (Table II). The C3-S4 and C3-S5 complexes consisted of two polypeptide chains, Arg55–Asn69 and Val110–Cys113, with heterogeneous cleavage at the C terminus of Leu119. Because these complexes contained only two cysteines, another direct disulfide assignment of Cys67–Cys113 was obtained. The C3-S6 and C3-S7 complexes had six cysteines in three polypeptide chains, Thr52–Leu64, Phe116–Glu133, and Leu154–Ala167, with heterogeneous cleavage at the C terminus of Val119. Because the yield for C3-S6 and C3-S7 complexes was too low for either further protease cleavage experiments or partial reduction and alkylation analysis, the C1 complex from the CNBr fragmentation was digested with subtilisin using a 1:9 (w/w) enzyme-to-substrate ratio, followed by RP-HPLC separations optimized to isolate the peptide complexes corresponding to C3-S6 and C3-S7 (described above) from this more complex starting sample. The purified peptide complex containing Thr52–Leu64, Phe116–Glu133, and Leu154–Ala167 from RP-HPLC of the first C1 subtilisin digestion (C1-S) was redigested with subtilisin (E:S = 1:3 (w/w)) to further fragment this complex. The C1-S-S1 complex had two cysteines in two polypeptide chains, Arg129–Glu133 and Leu154–Asp157, giving a direct disulfide assignment of Cys132–Cys156 (Table II). The C1-S-S1 showed a 43 Da mass increase compared with the expected sequence mass, and the N terminus was not available for Edman sequencing. These results suggest that the N-terminal amino group was carbamoylated. Apparently the extended incubation of this peptide in urea-containing buffers through multiple sequential protease digestions resulted in this artifactual modification. The C1-S-S2 peptide complex had four cysteines in disulfide linked peptide chains, Thr52–Leu64, Cys120–Cys128, and Lys158–Cys165. Because additional proteolysis of C1-S-S2 was not successful, partial reduction with TCEP and alkylation were used to complete disulfide bond assignments of this domain. The results from MALDI-MS and Edman sequence analyses of C1-S-S2 partial reduction and alkylation are summarized in Table III. Peptide C1-S-S2-R1 was the completely reduced and alkylated peptide Thr52–Leu64. C1-S-S2-R2 was composed of peptide Cys120–Cys128 with an alkylated cysteine and the peptide Lys158–Cys165. Edman sequencing of C1-S-S2-R1 showed that Cys120 was alkylated, indicating that this peptide complex was linked by the disulfide bond Cys128–Cys165 and that the remaining disulfide bond linkage was Cys57–Cys120. Therefore, the complete disulfide bond assignments of the sFRP-1 N-terminal Fz CRD was determined to be Cys67–Cys120, Cys67–Cys113, Cys104–Cys139, Cys126–Cys165, and Cys132–Cys156.

N-Linked Glycosylation Site of sFRP-1—The location and approximate size of the N-linked glycosylation site of sFRP-1 were determined by N-terminal Edman sequencing and MALDI-MS analyses of CNBr and subtilisin-digested peptide complexes and reduced peptides. As mentioned above, the CNBr C2 peptide complex consisted of three peptides, Lys211–Met270, Lys301–Phe312, and Thr169–Met210, with a mass 2812 Da higher than the expected amino acid sequence mass (Table I). This peptide complex contained the two potential N-linked glycosylation sites, Asn172 and Asn262. MALDI-MS analysis of the reduced C2 complex identified Lys211–Met270 with the ex-
Post-translational Processing of sFRP-1

TABLE II
MALDI-MS and N-terminal sequence analyses of subtilisin-digested peptide complexes
Data were obtained by MALDI-MS and N-terminal sequencing for the major subtilisin-digested peptide complexes isolated by RP-HPLC before and after reduction with TCEP.

| Subtilisin digestion | Assignment | Peptide massa | Comments |
|----------------------|------------|---------------|----------|
| C2–S1                | (202CAS)404, (202KHECFTPQ)309 | 1380.6 | 1380.6 | Cys202–Cys305 |
| C2–S1-reduced        | (202KHECFTPQ)309 | 1103.6 | 1103.5 | |
| C2–S2                | (139GT [TVCPFCDNHELK]193) | 3014.6 | 3012.3 | Heterogeneous cleavage |
|                      | (949LK | N | G | ADCPCHQLDLN)263 | 2442.2 | 2442.0 |
|                      | (164T-K)263 | 2499.2 | 2499.0 | |
|                      | (164T-K)263 | 2613.2 | 2613.1 | |
|                      | (164G-K)263 | 2901.2 | 2800.1 | |
|                      | (164G-K)263 | 2857.3 | 2657.1 | |
| C2–S2-reduced        | (164G-K)263 | 2771.3 | 2771.2 | |
| C2–S3                | (164G-K)263 | 1376.7 | 1376.6 | |
|                      | (164T-K)263 | 1218.6 | 1218.5 | |
|                      | (249L-L)263 | 1640.8 | 1640.7 | |
|                      | (23N-N)263 | 1399.7 | 1399.6 | |
|                      | (253G-L)263 | 1285.6 | 1285.5 | |
|                      | (253A-A)263 | 1228.5 | 1228.5 | |
| C2–S3-reduced        | (164G-K)263 | 2854.4 | 2854.2 | |
|                      | (164T-K)263 | 1376.7 | 1376.6 | |
|                      | (249L-L)263 | 1640.8 | 1640.7 | |

Subtilisin digestion of N-terminal CRD

C3–S2                | (139CHGTPQ)99 | 1143.5 | 1143.5 | Cys104–Cys139 |
|                      | (139CEPVMP14) | 616.3 | 616.2 | |
| C3–S3                | (139CQ909 | 1600.7 | 1600.7 | Cys104–Cys139 |
|                      | (139CEPVMP14) | 616.3 | 616.2 | |
|                      | (139CQ909 | 957.5 | 957.5 | |
|                      | (139CEPVMP14) | 1671.6 | 1674.1 | |
|                      | (139CQ909 | 616.2 | 616.2 | |
|                      | (139A-M142 | 1058.5 | 1058.9 | |
| C3–S4                | (66CHN173-{(110VFLC113) | 851.4 | 851.4 | Cys67–Cys113 |
|                      | (66C-N93 | 373.1 | 373.1 | |
| C3–S5                | (66LCHN174-{(120V-C113) | 1120.8 | 1120.5 | Cys67–Cys113 |
|                      | (66R-N93 | 642.5 | 642.3 | |
|                      | (66R-C13 | 812.0 | 812.0 | |
| C3–S6                | (66TTPQVDPAD61 | 4969.0 | 4983.2 | |
|                      | (132CLDRPIPCRMLC133- [154LKCDKPEGDVCIA167 | 1396.5 | 1396.7 | |
|                      | (132CLDRPIPCRMLC133- [154LKCDKPEGDVCIA167 | 1766.6 | 1766.8 | |
|                      | (132CLDRPIPCRMLC133- [154LKCDKPEGDVCIA167 | 1537.5 | 1537.7 | |
| C3–S7                | (132L-A67 | 5110.7 | 5079.4 | |
|                      | (132L-A67 | 1396.1 | 1396.7 | |
| C3–S7-reduced        | (132L-A67 | 2180.0 | 2181.0 | |
|                      | (132L-A67 | 1537.1 | 1537.7 | |

Subtilisin digestion of incomplete CNBr fragments

C1–S–S1             | (134RWLCE133- [154LKCD137 | 1224.5 | 1181.5 | Carboxamoylation on L154 (Δ = 43 Da) |
| C1–S–S1-reduced     | (134RWLCE133- [154LKCD137 | 706.3 | 706.3 | Cys137–Cys156 |
|                      | (134RWLCE133- [154LKCD137 | — | 521.2 | Not observed |
| C1–S–S2             | (134L-A67 | 3363.7 | 3364.6 | |
| C1–S–S2-reduced     | (134L-A67 | 1396.7 | 1396.7 | |
|                      | (134L-A67 | 1079.5 | 1079.5 | |
|                      | (134L-A67 | 894.5 | 894.4 | |

a Monoisotopic masses of single-charged molecules (MH)+.

b Down arrows indicate heterogeneous cleavage sites.

c C2–S3 and C1–S–S2 were used for partial reduction with TCEP and alkylation experiments.

d Assignments were confirmed by N-terminal sequencing.

e Data from peptide fractions that duplicate results from C2 and C3 are not shown for C1.

Expected mass, indicating that Asn262 was not modified. However, a mass for Thr169–Met210 which includes the potential N-linked glycosylation site at Asn172 was not observed. Instead, a weak and broad 7266-Da mass was observed, which suggested that Thr169–Met210 contained an ~2812 Da carbohydrate moiety on Asn172. Glycosylation at Asn172 was confirmed by Edman sequencing of peptides from the reduced C2 complex and C2–S3–R4 complex. The expected yield of Asn was observed at residue 262, indicating no apparent modification at this site. In contrast, no Asn was observed at residue 172, indicating that this Asn was completely modified. No evidence of O-linked glycosylation was observed in MALDI-MS analysis of CNBr and subtilisin fragments.

In addition to the above analyses, site-directed mutagenesis was performed on both possible N-linked glycosylation sites, Asn172 and Asn262, individually and simultaneously. Purified recombinant proteins containing either one or both of these substitutions were analyzed by SDS-PAGE, and their mobili-
ties were compared with that of wild-type sFRP-1 (Fig. 6). Derivatives containing the Gln172 substitution migrated faster than native sFRP-1, whereas the Gln262 modification did not alter the mobility of the proteins. These findings were consistent with the conclusions from MALDI-MS and Edman sequence analyses that N-linked glycosylation was present at Asn172 but not at Asn262.

DISCUSSION

The disulfide bonds in recombinant sFRP-1 have been determined by a combination of MALDI-MS, peptide mapping, and N-terminal sequencing. All peptide cleavage steps were carried out below pH 6.5 to prevent disulfide scrambling. The disulfide-bonding linkages in sFRP-1 are summarized in Fig. 2. CNBr treatment was chosen for the initial fragmentation in this study because attempts to cleave sFRP-1 with various proteases were not successful. Assignments of disulfide linkages in the C2-S3 (Gly181–Lys193–Leu249–Leu263) and C1-S-S2 (Thr52–Leu64–Cys120–Cys128–Lys158–Cys165) peptide complexes were not straightforward because each complex contained four cysteines (Table II). Because both peptide complexes were resistant to further proteolysis under all conditions evaluated, partial reduction with TCEP followed by alkylation was then used to determine disulfide bond assignments. Edman sequencing of the partially reduced and alkylated peptide complexes (C2-S3-R4 and C1-S-S2-R2) allowed the unambiguous assignment of these disulfide linkages, and no disulfide scrambling was observed.

The N-terminal portion of sFRP-1 has been predicted to be homologous to the putative Wnt-binding site of Frizzleds (38, 39). N-terminal sequencing of partially reduced and alkylated peptide complexes isolated by RP-HPLC.

| Peptide fraction | Assignment | Peptide mass | Comments |
|------------------|------------|-------------|----------|
|                  |            | Observed    | Calculated |       |
| Partial reduction and alkylation of C-terminal heparin binding domain | C2-S3-R1 | [183TVC*PPC*DNELK193] | 1332.3 | 1332.5 | Completely reduced and alkylated 183T–K193 |
|                  | C2-S3-R2 | [183GTTVC*PPC*DNELK193] | 1489.2 | 1490.6 | Completely reduced and alkylated 183G–K193 |
|                  | C2-S3-R3 | [249LKNGADC*PC*HQLDNL263] | 1754.7 | 1754.7 | Completely reduced and alkylated 249L–L263 |
|                  | C2-S3-R4 | [2973.4] | 2973.4 | 2972.3 | Partially reduced alkylated peptide complex Cys185–Cys255, Cys188–Cys257 |
| Partial reduction and alkylation of N-terminal CDR | C1-S2-R1 | [252TKPPQC*VDIPADL64] | 1455.9 | 1454.6 | Completely reduced and alkylated 52T–L64 |
|                  | C1-S2-R2 | [120C*LDRPIYPC128] | 2028.9 | 2027.9 | Partially reduced alkylated peptide complex Cys57–Cys120, Cys128–Cys165 |

* C* indicates carboxyamidomethylation of cysteine.
* Data for C2-S3-R4, C1-S2-R1, and C1-S2-R2 are average masses of single-charged molecules (MH+), whereas other values are monoisotopic masses.
* Calculated mass of each peptide includes mass of carboxyamidomethylation (number of carboxyamidomethylated residues × 58 Da).
* Assignments were confirmed by N-terminal sequencing.
* C2-S3-R4 has heterogeneity on N terminus of (181GT)183T–K193 that was not resolved by the RP-HPLC gradient used here. Mass heterogeneity caused by the heterogeneous cleavage indicated in parenthses.
39. The disulfide linkages and cysteine spacings of human sFRP-1 determined experimentally in the present study are compared with putative homologous domains of other proteins in Fig. 7. As shown, the sFRP-1 N-terminal Fz CRD module has a disulfide linkage pattern of 1–5, 2–4, 3–8, 6–10, and 7–9, consistent with the disulfide-bonding pattern of the Fz module recently determined in rat Ron1 receptor tyrosine kinase, mouse sFRP-3, and mouse Fz8 (49, 50). The cysteine spacings of these domains are highly conserved throughout the homologs and orthologs with the greatest variation occurring between C8 and C9 (spacing ranges from 12 to 27 residues) and intermediate variability between C2 and C4 (36–41 residues) and C5 and C10 (8–13 residues). However, it is quite interesting that Sizzled, Sizzled2, and Crescent, a subset of sFRPs that currently have been described only in Xenopus and chicken, contain an 11th cysteine residue (C11) in their CRDs, which is located between the conserved C8 and C9 residues (see below for further discussion and Fig. 7, upper panel). Diversity in these regions may contribute to distinct specificities for Wnt binding that presumably are characteristic of different Fz family members.

The disulfide linkages of the C-terminal domain of sFRP-1 determined experimentally in the present study are 1–4, 2–5, and 3–6. These assignments experimentally verify a primary disulfide linkage/cysteine spacing pattern (pattern A in Fig. 7) that was previously predicted in a model of NTR modules (49). As illustrated in Fig. 7, the sFRP-1 disulfide linkage matches that determined for human TIMPs (pattern B), although the location of C0 in the aligned sequences is quite different. Indeed, both cysteine spacings and disulfide linkages appear to be variable within putative NTR domains. We propose that NTR modules could be categorized into five groups or subfamilies based upon the divergent cysteine spacings and experimentally determined or predicted disulfide linkages (Fig. 7).

With the results for sFRP-1 described herein, assignments have now been rigorously determined for three of these five groups. The disulfide-bonding pattern of the sFRP-1 NTR domain most closely matches that of hNet2L1, the hPCOLCE1s, and hWFIFKKN (pattern A). Although relatively little is known about the functional significance of NTR domains, it is noteworthy that naturally occurring truncated fragments of hPCOLCE1 that begin slightly upstream of the NTR domain have 11 cysteines in their N-terminal Fz CRDs and only 5 cysteines in their C-terminal domains (Fig. 7, NTR pattern E). As noted above, they have an additional cysteine between C8 and C9 in the Fz CRD, whereas C5 has been lost. The conservation of the closely spaced cysteines, C1–C2 and C4–C5 and comparison with pattern A, suggests that the novel C0 might form a disulfide bond with the otherwise unpaired C2 (Fig. 7).

Furthermore, sFRP-4 contains two additional cysteine residues downstream of C5 that might form a disulfide bridge with each other. If NTR modules have functional significance for sFRPs, we surmise that the differences observed in the cysteine spacing and inferred disulfide bonding patterns would result in contrasting activities among the various family members.

Sizzled, Sizzled-2, and Crescent represent another subset of sFRPs with a potentially unique disulfide linkage pattern that affects both their Fz CRD and NTR domains. Specifically, they have 11 cysteines in their N-terminal Fz CRDs and only 5 cysteines in their C-terminal domains (Fig. 7, NTR pattern E). As noted above, they have an additional cysteine between C8 and C9 in the Fz CRD, whereas C5 has been lost from the C-terminal domain (compare pattern E versus A). Inspection of the recently determined mouse sFRP-3 Fz CRD crystal structure (51) together with alignment of the Sizzled and Crescent CRD sequences to the mouse sFRP-3 sequence strongly suggest the additional Sizzled/Crescent CRD cysteine is located on the surface of the CRD (Fig. 8). Cysteines exposed on surfaces of extracellular proteins usually form disulfide bonds due to the oxidizing extracellular environment. Hence, it is tempting to hypothesize that the NTR domain C2, which presumably would be unpaired due to the loss of C5, might form an interdomain disulfide bond with the additional, unpaired 11th cysteine located between C5 and C9 in the Fz CRD (Fig. 8). Of course this interesting model is highly speculative, but it also is readily testable. One alternative to the hypothesized inter-domain disulfide might be interchain disulfide bonds to yield covalent homodimers. The crystal structures of mouse sFRP-3 and mouse Fz8 showed Fz CRDs form non-covalent dimers under certain conditions. However, an intermolecular disulfide between the two 11th CRD cysteines in a dimer is not likely because the dimer interface in the crystal structure is on the opposite side of the molecule.

Previous reports indicated that sFRP biosynthesis was associated with partial proteolysis (36, 47). For instance, when epitope tags were placed at the C terminus of Xenopus Frzb-1/sFRP-3, the expected tagged proteins were detected in the intracellular compartment but not in the conditioned medium, implying proteolytic cleavage near the C terminus (36). In the present study, the predominant proteolytic event at the C terminus is removal of the terminal residue, Lys313. In addition, the N terminus was heterogeneously processed with the majority of purified sFRP-1 starting at Ser31 as well as minor amounts of protein starting with Asp41 or Phe60, as noted earlier (47). As described above, the unreduced protein was
FIG. 7. Cysteine spacing and disulfide bonding patterns of cysteine-rich motifs related to sFRP-1. Top panel (Fz CRD), experimentally determined disulfide structures (solid lines connecting cysteines) and cysteine spacings of human (h) sFRP-1 (this study) and rat Ror-1 (rRor-1) (50) are separately compared with closely related homologs described in a recent phylogenic study (32). Proteins represented include the following: human sFRP-3, -4, and -5 and mouse sFRP-1, -2, and -3 (sFRPs human and mouse); human Frizzled 3 and 5 (hFzd3,5); human muscle-specific kinase (hMuSK); human carboxypeptidase Z (hCPZ); human collagen XVIII isoform (hCollagen). The Fz CRD for the more divergent Xenopus Sizzled and Sizzled2 (Sz1 and Sz2) and chicken Crescent (Crescent) proteins that have an 11th cysteine are shown in a third group. Bottom panel (NTR Domain), five patterns of disulfide linkages based on experimental data (solid lines) and/or cysteine spacing are shown. Pattern A includes the disulfide assignments for human sFRP-1 determined in the present study; other proteins with similar cysteine spacings and presumably the same disulfide structure include the following: human sFRP-5; mouse sFRP-1,2; human netrin-2 like protein (hNet2l); human procollagen C-proteinase enhancer proteins-1 and -2 (hPCOLCE1,2); human WAP, Fs, Ig, Ku, and NTR protein (hWFIKKN) (49, 56, 57). Pattern B, experimentally determined disulfide structure for human tissue inhibitor of metalloproteinases 1 and 2 (hTIMP1,2) and predicted for human TIMP 3 and 4 (hTIMP3,4) (58, 59). Pattern C, experimentally determined disulfide structure for complement C3 and C4 and C5 (Complement C3,5). Pattern D, predicted disulfide structure (dotted lines connecting cysteines) for human sFRP-3 and -4 based on comparison of cysteine spacings with that of sFRP-1,2,5 as well as sequence alignment to human netrin 4 (see text). Pattern E, predicted disulfide structure (dotted lines) for Xenopus Sizzled1 and -2 (Sz1 and Sz2) and chicken Crescent (Crescent) based on comparison of cysteine spacings with sFRP-1,2,5. Putative unpaired cysteine is boxed. Cysteines in nonstandard locations (based on previous alignments (49)) are designated with C* or C#.
highly resistant to protease digestion with the exception of these small segments at both termini. A particularly protease-resistant core was identified as the disulfide linked [Phe50–Lys153]-[Asn51–Arg272] complex produced by extended trypsin digestion using high enzyme ratios in the presence of buffers containing 3 M urea (data not shown).

sFRP-1 has two potential N-linked glycosylation sites on Asn172 and Asn262 (Fig. 2). MALDI-MS and Edman sequence analyses of the C2 and C2-S3-R4 peptide complexes showed that Asn172 is completely glycosylated with a carbohydrate moiety of about 2812 Da, and Asn262 is not modified. Results from site-directed mutagenesis of both possible N-linked glycosylation sites were consistent with N-linked glycosylation at Asn172, and no evidence of glycosylation was observed at Asn262. The mass of the carbohydrate on Asn172 is ~200 Da less than the ~3000 Da difference between the calculated mass of the sFRP-1 sequence and the single peak observed in MALDI-MS of intact protein. This minor discrepancy is probably due to errors in the mass measurements caused by both heterogeneity of the carbohydrate moiety and the N terminus. However, there is a slight possibility an additional post-translational modification of the protein exists that eluded detection in the present study.

In conclusion, we have determined the disulfide linkages and glycosylation sites in human sFRP-1. The disulfide-bonding pattern of the N-terminal Fz CRD matches that recently reported for several other members of the Fz/sFRP family, whereas the pattern in the C-terminal domain reinforces the credibility of the NTR module as a structural entity. This assignment of disulfide linkages in a complete sFRP protein containing both a CRD domain and an NTR module should serve as the basis for exploring disulfide bond shuffling in the sFRP family. The variations in cysteine patterns within subsets of sFRPs suggest an unusual fluidity of disulfide bonds, which are typically strictly conserved over very wide evolutionary distances. Finally, the systematic analysis of the sFRP-1 post-translational modifications provides a sound basis for further structural and functional studies of this protein.

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REFERENCES

1. Dale, T. C. (1998) Biochem. J. 329, 209–223
2. Polakis, P. (2000) Genes Dev. 14, 1837–1851
3. Wang, Y., Macke, J. P., Abella, B. S., Andreasen, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Nathans, J. (1996) J. Biol. Chem. 271, 4468–4476
4. Wehrli, M., Dugan, S. T., Caldwell, K., O’Keefe, L., Schwartz, S., Vaizel-Ohyon, D., Schejter, E., Tomlinson, A., and DiNardo, S. (2000) Nature 407, 527–530
5. Tamai, K., Semenov, M. Kato, Y., Spokony, R., Liu, C., Catusayama, Y., Hess, F., Saint-Jeannet, J.-P., and He, X. (2000) Nature 407, 530–535
6. Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J., and Skarnes, W. C. (2000) Nature 407, 535–538
7. Brown, M. S., Herz, J., and Goldstein, J. L. (1997) Nature 388, 629–630
8. Brown, S. D., Twells, R. C. J., Hey, P. J., Cox, R. D., Levy, E. R., Soderman, A. B., Metzker, M. L., Caskey, C. T., Todd, J. A., and Hess, J. F. (1998) Biochem. Biophys. Res. Commun. 249, 879–888
9. Zeng, L., Fagotto, F., Zhang, T., Heu, W., Vasiek, T. J., Perry, W. L., Lee, J. J., Tilghman, S. M., Gumbiner, B. M., and Costantini, F. (1997) Cell 96, 181–192
10. Behrens, J., Jerchow, B. A., Wurtele, M., Grinn, J., Ashbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birnichmeier, W. (1998) Science 280, 596–599
11. Ikeda, S., Kishida, S., Yamamoto, H., Marui, H., Koyama, S., and Kikuchi, A. (1998) EMBO J. 17, 1371–1384
12. Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S., and Kikuchi, A. (1998) J. Biol. Chem. 273, 10823–10826
13. Sakanaka, C., Weiss, J. B., and Williams, L. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3020–3023
14. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) EMBO J. 16, 3797–3804
15. Orford, K., Crockett, C., Jensen, J. P., Weissman A. M., and Byers, S. W. (1997) J. Biol. Chem. 272, 24735–24738
16. Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., and Kikuchi, A. (1999) Mol. Cell. Biol. 19, 4414–4422
17. Li, L., Yuan, H., Weaver, C. D., Mao, J., Farr, G. H., Sussman, D. J., Jonsker, J., Kimmelman, D., and Wu, D. (1999) EMBO J. 18, 4233–4240
18. Smallay, M. J., Sara, E., Paterson, H., Nayler, S., Cook, D., Jayati, H., Fryer, L. G., Hutchinson, L., Fry, M. J., and Dale, T. C. (1999) EMBO J. 18, 2823–2835
19. Ihn, K., Antipova, A., Ratcliffe, M. J., and Sokol, S. (2000) Mol. Cell. Biol. 20, 2228–2238
20. Farr, G. H., Ferkey, D. M., Yost, C., Pier, S. B., Weaver, C., and Kimelman, D. (2000) J. Cell Biol. 148, 691–702
21. Dolmer, K., and Sottrup-Jensen, L. (1993) FEBS Lett. 351, 85–90
22. Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S., and Kikuchi, A. (1999) J. Biol. Chem. 274, 10681–10684
23. Willert, K., Shihabmoto, S., and Nuzze, R. (1999) Genes Dev. 13, 1768–1773
24. Mao, J., Wang, J., Liu, B., Pan, W., Farr, G. H., Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., and Wu, D. (2001) Mol. Cell 7, 801–809
25. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R.,
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26. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996) Cell 88, 391–399
27. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursch, D., Jones, T., Beissovec, A., Peifer, M., Mörten, M., and Clevers, H. (1997) Cell 88, 789–799
28. He, T. C., Sparks, A. B., Rago, C., Heremeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) Science 281, 1509–1512
29. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D’Amico, M., Pestell, R., and Ben-Ze’ev, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5522–5527
30. Tetsu, O., and McCormick, F. (1999) Nature 398, 422–426
31. Bhanot, P., Brink, M., Samos, C. H., Hsieh, J.-C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J., and Nusse, R. (1996) Nature 382, 225–230
32. Xu, Y. K., and Nusse, R. (1998) Curr. Biol. 8, R405–R406
33. Saldanha, J., Singh, J., and Mahadevan, D. (1998) Protein Sci. 7, 1632–1635
34. Song, L., and Fricker, L. D. (1997) J. Biol. Chem. 272, 10543–10550
35. Rehn, M., and Pihlajaniemi, T. (1995) J. Biol. Chem. 270, 4705–4711
36. Leyns, L., Bouwmeester, T., Kim, S. H., Piccolo, S., and De Robertis, E. M. (1997) Cell 88, 747–756
37. Wang, S., Brink, M., Lin, K., Layten, F. P., and Moos, M., Jr. (1997) Cell 88, 757–766
38. Rattner, A., Hsieh, J.-C., Smallwood, P. M., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Nathans, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2659–2663
39. Finch, P. W., He, X., Kelley, M. J., Uren, A., Schaudies, R. P., Popescu, N. C., Rudikoff, S., Aaronson, S. A., Varmus, H. E., and Rubin, J. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6770–6775
40. Sala, A. N., Kroll, K. L., Evans, L. M., and Kirschner, M. W. (1997) Development 124, 4739–4748
41. Melkonyan, H. S., Chang, W. C., Shapiro, J. P., Mahadevappa, M., Fitzpatrick, P. A., Kiefer, M. C., Tomei, L. D., and Umanysky, S. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1384–1390
42. Pfeffer, P. L., De Robertis, E. M., and Izpisua-Belmonte, J. C. (1997) Int. J. Dev. Biol. 41, 449–458
43. Wolf, V., Ke, G., Dharmarajan, A. M., Bielke, W., Artuso, L., Saurer, S., and Friis, R. (1997) FEBS Lett. 417, 385–389
44. Xu, Q., D’Amore, P. A., and Sokol, S. Y. (1998) Development 125, 4767–4776
45. Chang, J. T., Esumi, N., Moore, K., Li, Y., Zhang, S., Chew, C., Goodman, B., Rattner, A., Moody, S., Stetten, G., Campochiaro, P. A., and Zaid, D. J. (1999) Hum. Mol. Genet. 8, 575–583
46. Bradley, L., Sun, B., Collins-Racic, L., LaVallie, E., McCoy, J., and Sive, H. (2000) Dev. Biol. 227, 118–132
47. Uren, A., Reichman, F., Anest, V., Taylor, W. G., Muraiso, K., Bottaro, D. P., Cumberledge, S., and Rubin, J. S. (2000) J. Biol. Chem. 275, 4374–4382
48. Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessel, T. M., and Tessier-Lavigne, M. (1994) Cell 78, 409–424
49. Bányai, L., and Pathy, L. (1999) Protein Sci. 8, 1636–1642
50. Roszmusz, E., Pathy, A., Trexler, M., and Pathy, L. (2001) J. Biol. Chem. 276, 18485–18490
51. Dann, C. E., Hsieh, J.-C., Rattner, A., Sharma, D., Nathans, J., and Leahy, D. (2001) Nature 412, 86–90
52. Gray, W. R. (1993) Protein Sci. 2, 1732–1748
53. Reim, D. F., and Speicher D. W. (1997) in Current Protocols in Protein Science (Coligan J. E., Dunn, B. M., Fleshe, H. L., Speicher, D. W., and Wingfield, P. T., eds) pp. 11.10.1–11.10.38, John Wiley & Sons, Inc., New York
54. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Anal. Chem. 68, 850–858
55. Mott, J. D., Thomas, C. L., Rosenbach, M. T., Takahara, K., Greenspan, D. S., and Banda, M. J. (2000) J. Biol. Chem. 275, 1384–1390
56. Xu, H., Acott, T. S., and Wirtz, M. K. (2000) Genomics 66, 264–273
57. Trexler, M., Banyai, L., and Pathy, L. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3705–3709
58. Williamson, R. A., Marston, F. A., Angal, S., Koklitis, P., Panico, M., Morris, H. R., Carne, A. F., Smith, B. J., Harris, T. J., and Freedman, R. B. (1999) Biochem. J. 368, 267–274
59. Fernandez-Catalan, C., Bode, W., Haber, R., Turk, D., Calvete, J. J., Lichte, A., Tescsche, H., and Maskos, K. (1998) EMBO J. 17, 5238–5248