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Patente de Invención ☐

Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (Fase Nacional) ☐

Cap. I ☐

Cap. II ☐

No. Solicitud Internacional PCT/IB2007/002841

Fecha de Presentación Internacional 28 Septiembre 2007

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 13 a 42	08/Abril/2009
Reivindicaciones:	Folios 43 a 50	08/Abril/2009
	Folios 100 a 106	08/Abril/2009
Dibujos:	Folios 55 a 56	08/Abril/2009
Secuencias:	Folios 51 a 54	08/Abril/2009
Prioridad:	EP 06021521.7	13/Octubre/2006

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Tensioactivos reconstituidos que tienen propiedades mejoradas"

El problema planteado en esta solicitud consiste en la necesidad de suministrar un tensioactivo reconstituido que sea capaz de garantizar estabilidad alveolar y por lo tanto de mantener la permeabilidad alveolar al final de la espiración sin requerir ventilación con PEEP (Presión Positiva al final de la espiración).

Para resolver este problema técnico la solicitud en estudio proporciona un tensioactivo reconstituido que comprende un portador lípido y una combinación de un análogo de polipéptido particular de la proteína tensioactiva nativa SP-C, con un

Expediente 09 36247 1er Art 45

análogo de polipéptido particular de la proteína tensioactiva nativa SP-B.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C 07 K 14/785; A 61 K 38/16; A 61 P 11/00

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 19 y 20 no es patentable, de acuerdo con el Art 14.

5. De la Solicitud de Patente

Título

El título no corresponde con el objeto de la solicitud porque tanto en la descripción como en las reivindicaciones se divulga un tensioactivo pulmonar modificado que comprende un análogo de la proteína SP-C, un análogo de la proteína SP-B y una mezcla de fosfolípidos, por lo cual se sugiere al solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara porque reclama un tensioactivo pero este no corresponde a un único compuesto sino a una mezcla de compuestos. Se sugiere definir la invención como una composición tensioactiva. Adicionalmente, el término "reconstituido" hace referencia a una etapa de un proceso, como es la reconstitución, y no define las características técnicas esenciales del tensioactivo reclamado. Se sugiere excluir el término "reconstituido" o reemplazarlo por el más adecuado. Por otra parte, la reivindicación 1 no es clara porque la definición de Ω en la fórmula (I) incluye el término "oxidizado", el cual es poco reconocido en el campo técnico. Se sugiere definirlo correctamente. También se sugiere corregir el término "N" de la fórmula (I) por "n" y corregir el término "Sf" usado para definir el último residuo de aminoácido de la fórmula (II) por "S_f".

La reivindicación 1 no es concisa porque reclama una mezcla de fosfolípidos pero no se incluyen las opciones preferidas de los mismos. Se sugiere definir los fosfolípidos preferidos incluyendo su denominación común internacional o nombre genérico.

La reivindicación 3 no es concisa porque reclama una combinación fija de los análogos de SP-C y SP-B pero no se definen las proporciones que hacen parte de dicha combinación fija. Se sugiere incluir las proporciones en las que se encuentran los compuestos dentro de la composición.

Se sugiere restringir la solicitud a una razonable generalización del tipo de compuestos que ha sido sintetizado ó probado, es decir, que se encuentran debidamente sustentados. El péptido de fórmula (I) sustentado corresponde a IPSSPVHLKRLKLLLLLLLLLILLLILGALLLGL (If); donde e, n=0; a, f, g, p=1; b=14; X=L; B=K; y Ω = L.

La reivindicación 4 no es clara porque los puentes disulfuro del polipéptido de fórmula (II) reclamados no son posibles cuando f es 0. Se sugiere restringir el objeto de la invención al polipéptido de fórmula (II) cuando f es 1.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad (Octubre 13 de 2006) y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO0047623	Artificial peptides having surface activity and the use thereof in the preparation of artificial surfactant	17/Agosto/2000
D2	Waring AJ et al. The Journal of Peptide Research. 2005;66(6):364-74.	The role of charged amphipathic helices in the structure and function of surfactant protein B	Diciembre/2005

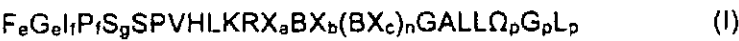
D1<http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=0047623A1&KC=A1&FT=D&ND=3&date=20000817&DB=EPODOC&locale=en> EP divulga (Pág. 1, líneas 1-9) análogos de SP-C los cuales, una vez combinados con lípidos adecuados, son efectivos en la reducción de la tensión superficial en la interface aire-líquido. Los péptidos de la invención también pueden ser combinados con SP-B, uno de sus análogos activos o un sustituto de SP-B.

D2<http://www.ncbi.nlm.nih.gov/pubmed?term=The%20role%20of%20charged%20amphipathic%20helices%20in%20the%20structure%20and%20function%20of%20surfactant%20protein%20B> divulga (Pág. 366, columna 1, párrafo 3, ver folio 161) el análogo de SP-B de fórmula NH₂-C₁WLCRALIKRIQAMIPKGGRMLPQLVCRLVLRCS₃₄-COOH.

7. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en un tensioactivo reconstituido que comprende una mezcla de fosfolípidos y una combinación de un análogo de polipéptido de la proteína tensioactiva nativa SP-C, con un análogo de polipéptido de la proteína tensioactiva nativa SP-B, caracterizado porque el análogo de polipéptido de la proteína tensioactiva nativa SP-C es un polipéptido de al menos 20 residuos de aminoácido y no más de 40 residuos de aminoácido que tienen la secuencia representada por la fórmula general (I)



y en donde el análogo de polipéptido de la proteína tensioactiva nativa SP-B, es un polipéptido de la fórmula general (II):



Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Un tensioactivo reconstituido que comprende:	Péptidos con actividad superficial que comprenden:	
Una mezcla de fosfolípidos	Fosfolípidos como DPPC (dipalmitoilfosfatidilcolina), PG (fosfatidilglicerol), etc,	Fosfolípidos como DPPC (dipalmitoilfosfatidilcolina), POPG (palmitoiloleilfosfatidilglicerol), etc.

Características esenciales	D1	D2
Un análogo de polipéptido de la proteína tensioactiva nativa SP-C, representado por la fórmula general (I): $F_eG_eI_fP_fS_gSPVHLKRX_aBX_b(BX_c)_nGALL\Omega_pG_pL_p$ donde S está opcionalmente sustituido con grupos acilo C_{12-22} $X = I, L$ o nL $B = K, W, F, Y$ u ornitina $\Omega = M$ $a = 1-8; b = 1-19; c = 3-8; e, f, g, p = 0$ ó $1; n = 0-3;$ $X_aBX_b(BX_c)_n$ tiene un máximo de 22 aminoácidos.	Análogos de SP-C de fórmula general (I): $F_eG_eIPZZPVHLKR(X_aB)(X_bB)_n(X_cB)_mX_dGALLMGL$ donde: $Z = S$ y S está opcionalmente sustituido con grupos acilo C_{12-22} $X = I, L$ o nL $B = K, W, F, Y$ u ornitina $a = 1-19; b = 1-19; c = 1-21; d = 0; e, f, n, m = 0$ ó 1 $(X_aB)(X_bB)_n(X_cB)_mX_d$ tiene un máximo de 22 aminoácidos.	
Un análogo de polipéptido de la proteína tensioactiva nativa SP-B, de fórmula general (II): $C_i\Delta LCRALIKRIQA\Omega IPKGG R\Omega LPQLVCRLVL\Phi CS_f$ donde $\Delta = W$ o L $\Omega = M, I, L$ o nL $\Phi = R$ o T $f = 0$ ó 1	SP-B o uno de sus análogos activos	Un análogo peptídico de la proteína tensioactiva SP-B, de fórmula: $NH_2-C_1WLCRALIKRIQAMIPKGGRMLPQLVCRLVLRCS_{34}-COOH$

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 divulga análogos de SP-C los cuales, una vez combinados con lípidos adecuados, son efectivos en la reducción de la tensión superficial en la interface aire-líquido. Los péptidos de la invención también pueden ser combinados con SP-B, uno de sus análogos activos o un sustituto de SP-B, para preparar surfactantes artificiales útiles en el tratamiento de síndrome de dificultad respiratoria neonatal (RDS) y otras deficiencias de surfactante (Pág. 1, líneas 1-9). D1 enseña análogos de SP-C de fórmula general (I): $F_eG_eI_fP_fS_gSPVHLKRX_aBX_b(BX_c)_nGALL\Omega_pG_pL_p$ (Pág. 5, líneas 23-26; reivindicación 1). En particular, los péptidos se han combinado con DPPC (dipalmitoilfosfatidilcolina), PG (fosfatidilglicerol), entre otros fosfolípidos (Pág. 8, líneas 1-4). D1 divulga que la adición de SP-B o uno de sus análogos activos a la mezcla de péptido/fosfolípidos otorga resultados particularmente favorables (Pág. 8, líneas 12-13; tabla 2).

D2 da a conocer el análogo de SP-B de fórmula $NH_2-C_1WLCRALIKRIQAMIPKGGRMLPQLVCRLVLRCS_{34}-COOH$, denominado péptido Mini-B oxidado (Pág. 366, columna 1, párrafo 3, ver folio 161). Al mezclar este péptido con fosfolípidos como DPPC (dipalmitoilfosfatidilcolina), POPG (palmitoiloleilfosfatidilglicerol), entre otros y medir la tensión superficial se encontró que la mezcla reproduce las funciones esenciales de la proteína SP-B de longitud completa tanto in vivo como in vitro (Pág. 367, columna 1, párrafo 3, ver folio 161;

Pág. 371, columna 2, párrafo 2, ver folio 163).

La diferencia entre la invención, definida en la reivindicación 1 y los péptidos de D1 consiste en que el análogo de SP-B tiene la fórmula general $C_7\Delta LCRALIKRIQA\Omega IPKGGR\Omega LPQLVCRLVL\Phi CS_r$.

El hecho de incluir el análogo del péptido SP-B de fórmula general $C_7\Delta LCRALIKRIQA\Omega IPKGGR\Omega LPQLVCRLVL\Phi CS_r$ en la mezcla compuesta por el análogo de SP-C y fosfolípidos no muestra un efecto técnico mejorado frente a la estabilidad alveolar.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar los péptidos con actividad superficial conocidos en D1 para conseguir tensioactivos alternativos con actividad similar frente a la estabilidad alveolar”.

Sin embargo, la utilización del análogo del péptido SP-B de fórmula general $C_7\Delta LCRALIKRIQA\Omega IPKGGR\Omega LPQLVCRLVL\Phi CS_r$ para reducir la tensión superficial en los alvéolos, ya está divulgada en el documento D2 que se refiere al análogo de SP-B denominado péptido Mini-B oxidado de fórmula general como la mencionada anteriormente (Pág. 366, columna 1, párrafo 3, ver folio 161). Al mezclar este péptido con fosfolípidos como DPPC (dipalmitoilfosfatidilcolina), POPG (palmitoiloleilfosfatidilglicerol), entre otros y medir la tensión superficial se encontró que la mezcla reproduce las funciones esenciales de la proteína SP-B de longitud completa tanto in vivo como in vitro (Pág. 367, columna 1, párrafo 3, ver folio 161; Pág. 371, columna 2, párrafo 2, ver folio 163).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el análogo del péptido SP-B de fórmula general $C_7\Delta LCRALIKRIQA\Omega IPKGGR\Omega LPQLVCRLVL\Phi CS_r$ de D2 en el tensioactivo de D1 para llegar así al objeto de la reivindicación 1 de la solicitud, por lo cual la reivindicación 1 se considera obvia.

Puesto que las reivindicaciones dependientes 2 a 18 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:
Las reivindicaciones 1 a 18 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

8. Conclusión

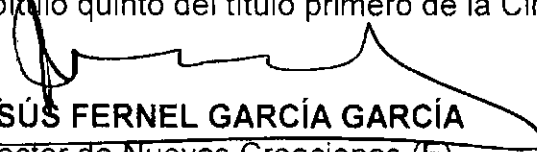
Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO0047623	1 – 18	Nivel Inventivo
D2	Waring AJ et al. The Journal of Peptide Research. 2005;66(6):364-74.	1 – 18	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de

no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JESÚS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha

~~14 8 JUL. 2012~~

Elaborado por: Mónica Alexandra Nova Manosalva
Revisado por: Consuelo Leguizamón Leguizamón
Aprobado por: Jesús Fernel García García



The role of charged amphipathic helices in the structure and function of surfactant protein B

detectors; ^{13}C FTIR, conventional FTIR, spectroscopy; CD, circular dichroism; 2D-TAMs, two-dimensional nuclear magnetic resonance; ESI, electrospray ionization; HPLC, high performance liquid chromatography; K_2S , synthetic π -helical intrinsic of 51.6; 21-residues; Mm-B, BPH; CH₂CL₂/RIDA/MA/GG/MA/POLYLYC/COOH, 34-residue; Mm-B/MA, 14H₂-GG/MA/TT/GA/MA/MA/TT/GG/MA/POLYLYC/TS.

Key words: amphipathic helices; captive bubble surfactometry;

isotopu-enhanced Fourier transform infrared spectroscopy; lepidote; saposin; secondary structure; surfactant activity

Abstract: Surfactant protein B (SP-B) is essential for normal lung surfactant function. Theoretical models predict that the disulfide cross-linked, N- and C-terminal domains of SP-B fold as charged amphipathic helices and suggest that these adjacent helices

sequences of the N- and C-terminal domains. Consistent with using a disulfide-linked construct (Mm-8) based on the primary participate in critical surfactant activities, this hypothesis is tested

theoretical predictions of the full length protein, both isotope-enhanced Fourier transform infrared (FTIR) spectroscopy and molecular modeling confirm the presence of charged amphipathic

8 in model surfactant lipid mixtures exhibits marked *in vitro*

activity, with spread films showing near-zero minimum surface tension during cycling using capillary bubble surfaceometry. In vivo, Mini-B shown oxygenation and dynamic compliance that compare favorably with that of full-length SpB. Mini-B variants (i.e., reduced disulfides or cationic residues replaced by uncharged residues) or Mini-B fragments (i.e., unlinked N- and C-terminal domains) produced greatly attenuated in vivo and in vitro surfactant monolayers. These data indicate that the combination of surfactant monolayers and spread films is a powerful tool for studying the function of surfactant proteins.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; POPE, palmitoleoyllecithin; cardiolipin, POPE, palmitoleoylphosphatidylethanolamine; Chol, cholesterol; POH, palmitoleoylphosphatidylol; PPS, 1-phosphatidyl-1'-phosphatidylcholine; LIPF, hexafluoroisopropylidene; ATR, attenuated total reflectance; FTIR, Fourier transform infrared spectroscopy; ^{13}C FTIR, isotopically enhanced ^{13}C Fourier transform infrared

peptide representing the N-terminal domain of SP-B (i.e., SP-B₁₋₃₇ residues) each increase the collapse pressure of lipid monolayers containing palmitic acid. This suggests that the cationic N-terminus of SP-B interacts with anionic lipids to remove the driving force for lipid squeeze-out from lipid bilayers.

the surface film [44,25]. The critical insertion pressure, and index of the degree of protein association with lipid films, is also very high for both $\text{Si}^{\text{R}}\text{H}_{12}$ and the parent $\text{Si}^{\text{R}}\text{H}_{18}$ [21,26,27]. Moreover, $\text{Si}^{\text{R}}\text{H}_{12}$ and (n)-length $\text{Si}^{\text{R}}\text{H}$ chains induce a coexistence of buckled and flat monolayers when added to surfactant lipids, leading to a low minimum surface tension and increased spreading of the surfactant monolayer [25,26]. These *in vitro* surfactant activities of $\text{Si}^{\text{R}}\text{H}_{12}$ are correlated with the improved oxygenation and air compliance observed with this peptide in surfactant-treated premature newborns.

deficient animal models (10–12). As physical experiments indicate that N-terminal SP-B peptides in lipids or membranes show high α -helix content (26,30,31), the N-terminal domain of SP-B may participate in its in vitro and in vivo surfactant activities as a charged amphipathic α -helix. Interestingly, several non-natural analogs of SP-B, which form α -helices in lipid environments also exhibit in vitro surfactant activities (32). A charged helical C-terminal domain of SP-B may similarly participate in lung function, as synthetic peptides based on the C-terminal domain assume helical conformations (33,34) and also induce in vitro surfactant activities (35) and in vivo (13,14) surfactant activities that simulate those of the native protein. $K_{1/2}$, a synthetic 21-residue peptide which more generally mimics the amphipathic and hydrophilic repeats of SP-B, may achieve its surfactant activities (14,36) by folding as an α -helix (17);

In the present study, Mini-R, a synthetic construct (14-residue) based on the N- and C-terminal regions and the disulfide cross-linkages of native S1-B, was shown via isotope enhanced FTIR spectroscopy and molecular modeling to share the conformational organization of these-

1862, 1863, 1864, 1865, 1866, 1867, 1868, 1869, 1870, 1871, 1872, 1873, 1874, 1875, 1876, 1877, 1878, 1879, 1880, 1881, 1882, 1883, 1884, 1885, 1886, 1887, 1888, 1889, 1890, 1891, 1892, 1893, 1894, 1895, 1896, 1897, 1898, 1899, 1900, 1901, 1902, 1903, 1904, 1905, 1906, 1907, 1908, 1909, 1910, 1911, 1912, 1913, 1914, 1915, 1916, 1917, 1918, 1919, 1920, 1921, 1922, 1923, 1924, 1925, 1926, 1927, 1928, 1929, 1930, 1931, 1932, 1933, 1934, 1935, 1936, 1937, 1938, 1939, 1940, 1941, 1942, 1943, 1944, 1945, 1946, 1947, 1948, 1949, 1950, 1951, 1952, 1953, 1954, 1955, 1956, 1957, 1958, 1959, 1960, 1961, 1962, 1963, 1964, 1965, 1966, 1967, 1968, 1969, 1970, 1971, 1972, 1973, 1974, 1975, 1976, 1977, 1978, 1979, 1980, 1981, 1982, 1983, 1984, 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2029, 2030, 2031, 2032, 2033, 2034, 2035, 2036, 2037, 2038, 2039, 2040, 2041, 2042, 2043, 2044, 2045, 2046, 2047, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2061, 2062, 2063, 2064, 2065, 2066, 2067, 2068, 2069, 2070, 2071, 2072, 2073, 2074, 2075, 2076, 2077, 2078, 2079, 2080, 2081, 2082, 2083, 2084, 2085, 2086, 2087, 2088, 2089, 2090, 2091, 2092, 2093, 2094, 2095, 2096, 2097, 2098, 2099, 2100, 2101, 2102, 2103, 2104, 2105, 2106, 2107, 2108, 2109, 2110, 2111, 2112, 2113, 2114, 2115, 2116, 2117, 2118, 2119, 2120, 2121, 2122, 2123, 2124, 2125, 2126, 2127, 2128, 2129, 2130, 2131, 2132, 2133, 2134, 2135, 2136, 2137, 2138, 2139, 2140, 2141, 2142, 2143, 2144, 2145, 2146, 2147, 2148, 2149, 2150, 2151, 2152, 2153, 2154, 2155, 2156, 2157, 2158, 2159, 2160, 2161, 2162, 2163, 2164, 2165, 2166, 2167, 2168, 2169, 2170, 2171, 2172, 2173, 2174, 2175, 2176, 2177, 2178, 2179, 2180, 2181, 2182, 2183, 2184, 2185, 2186, 2187, 2188, 2189, 2190, 2191, 2192, 2193, 2194, 2195, 2196, 2197, 2198, 2199, 2200, 2201, 2202, 2203, 2204, 2205, 2206, 2207, 2208, 2209, 2210, 2211, 2212, 2213, 2214, 2215, 2216, 2217, 2218, 2219, 2220, 2221, 2222, 2223, 2224, 2225, 2226, 2227, 2228, 2229, 2230, 2231, 2232, 2233, 2234, 2235, 2236, 2237, 2238, 2239, 2240, 2241, 2242, 2243, 2244, 2245, 2246, 2247, 2248, 2249, 2250, 2251, 2252, 2253, 2254, 2255, 2256, 2257, 2258, 2259, 2260, 2261, 2262, 2263, 2264, 2265, 2266, 2267, 2268, 2269, 2270, 2271, 2272, 2273, 2274, 2275, 2276, 2277, 2278, 2279, 2280, 2281, 2282, 2283, 2284, 2285, 2286, 2287, 2288, 2289, 2290, 2291, 2292, 2293, 2294, 2295, 2296, 2297, 2298, 2299, 2300, 2301, 2302, 2303, 2304, 2305, 2306, 2307, 2308, 2309, 2310, 2311, 2312, 2313, 2314, 2315, 2316, 2317, 2318, 2319, 2320, 2321, 2322, 2323, 2324, 2325, 2326, 2327, 2328, 2329, 2330, 2331, 2332, 2333, 2334, 2335, 2336, 2337, 2338, 2339, 2340, 2341, 2342, 2343, 2344, 2345, 2346, 2347, 2348, 2349, 2350, 2351, 2352, 2353, 2354, 2355, 2356, 2357, 2358, 2359, 2360, 2361, 2362, 2363, 2364, 2365, 2366, 2367, 2368, 2369, 2370, 2371, 2372, 2373, 2374, 2375, 2376, 2377, 2378, 2379, 2380, 2381, 2382, 2383, 2384, 2385, 2386, 2387, 2388, 2389, 2390, 2391, 2392, 2393, 2394, 2395, 2396, 2397, 2398, 2399, 2400, 2401, 2402, 2403, 2404, 2405, 2406, 2407, 2408, 2409, 2410, 2411, 2412, 2413, 2414, 2415, 2416, 2417, 2418, 2419, 2420, 2421, 2422, 2423, 2424, 2425, 2426, 2427, 2428, 2429, 2430, 2431, 2432, 2433, 2434, 2435, 2436, 2437, 2438, 2439, 2440, 2441, 2442, 2443, 2444, 2445, 2446, 2447, 2448, 2449, 2450, 2451, 2452, 2453, 2454, 2455, 2456, 2457, 2458, 2459, 2460, 2461, 2462, 2463, 2464, 2465, 2466, 2467, 2468, 2469, 2470, 2471, 2472, 2473, 2474, 2475, 2476, 2477, 2478, 2479, 2480, 2481, 2482, 2483, 2484, 2485, 2486, 2487, 2488, 2489, 2490, 2491, 2492, 2493, 2494, 2495, 2496, 2497, 2498, 2499, 2500, 2501, 2502, 2503, 2504, 2505, 2506, 2507, 2508, 2509, 2510, 2511, 2512, 2513, 2514, 2515, 2516, 2517, 2518, 2519, 2520, 2521, 2522, 2523, 2524, 2525, 2526, 2527, 2528, 2529, 2530, 2531, 2532, 2533, 2534, 2535, 2536, 2537, 2538, 2539, 2540, 2541, 2542, 2543, 25

domains in SP-B. Captive bubble surfactometry and animal models of respiratory distress indicate that the specific 3D structure and charge distribution of helices in Mini-B may be responsible for key *in vitro* and *in vivo* activities of full-length SP-B in lung surfactant.

Experimental Procedures

Materials

Lipide synthesis reagents, including *l*-proline, *l*-valine, and coupling solvents, were obtained from Applied Biosystems (Foster City, CA, USA). Deuterium oxide was supplied by Aldrich Chemical Co. (Milwaukee, WI, USA). ¹³C-*l*-leucine, *l*-leucine, *l*-isoleucine, and *l*-valine were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Dipalmitoylphosphatidylcholine (DPPC), palmitoleoylphosphatidylglycerol (POPG), dioleoylphosphatidylcholine (DOPC), palmitoylcholinephosphatidylcholine (PCPE), palmitoylcholinephosphatidylcholine (PCPE), and cholesterol (Chol; purity 99%) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Solid phase peptide synthesis, purification, and characterization

The oxidized Mini-B peptide (34-residue; NH₂-C¹⁵WL¹³RAIRKQAMVCGGIMFQVCEHVLVRC¹⁶-COOH) was synthesized using FastMoc™ (Applied Biosystems) chemistry with an ABI peptide synthesizer, and purified by reverse phase high performance liquid chromatography (HPLC; 38). Mini-B residues 1–18 correspond to the N-terminal SP-B residues 8–23 (i.e., SP-B_{8–23}) of the full-length human protein, while Mini-B residues 19–34 correspond to the C-terminal SP-B residues 63–78 (i.e., SP-B_{63–78}). The disulfide linkages were directed by using acid labile t-butyl protecting groups at cysteine positions 1 and 33, while acid resistant acetamidomethyl (ACAM) side chain-protecting groups were employed at cysteine positions 4 and 27. The disulfide bond between Cys-1 and -33 was formed by air oxidation for 24 h trifluoroethanol–ammonium bicarbonate buffer (10 mM, pH 8.0) followed by reduction of the ACAM protected cysteines. The disulfide connectivity was confirmed by mass spectrometry of the enzyme-digested fragments (trypsin, chromatography, Emory University Microchemical Facility). A reduced Mini-B peptide was similarly prepared, except that the oxidizing steps were omitted and cysteine residues 1 and 33 SH groups were

blocked with *N*-ethylmaleimide. Mini-B_{19–34}, an oxidized variant of Mini-B with threonine replacing positively charged residues, was synthesized and purified following the protocol for oxidized Mini-B, using the sequence: NH₂-C¹⁵WL¹³RAIRKQAMVCGGIMFQVCEHVLVRC¹⁶-COOH. The fragment peptides SP-B_{8–23} (N-terminal 18-residue; NH₂-C¹⁵WL¹³RAIRKQAMVCGGIMFQVCEHVLVRC¹⁶-COOH), numbered based on the full-length SP-B, were similarly synthesized. Purified native porcine SP-B protein was prepared [39] and identified by N-terminal sequence analysis (Emory University Microchemical Facility).

Fourier transform infrared spectroscopy

Infrared spectra were recorded at 25 °C using a Bruker Vector 22™ FTIR spectrometer (Bruker Optics, Billerica, MA, USA) with a DTEGS detector, averaged over 356 scans at a gain of 4 and a resolution of 2 per cm. For SP-B peptides, originally in 90% hexafluoroisopropanol (HFIH/90% D₂O), self-films were prepared by air drying peptide solutions onto a 50 × 50 × 2 mm, 45° attenuated total reflectance (ATR) crystal for the Bruker (JPK Technologies, Madison, WI, USA) spectrometer (40,41). The overall secondary conformations were qualitatively assessed from the amide I band of conventional ¹³C FTIR spectra of SP-B peptides (50,42). The frequency limits for the different structures were: α -helix (1652–1613 per cm), β -turns (1635–1602 per cm), and disordered or random (1650–1637 per cm, 42).

Rationale for ¹³C-isotope-directed Mini-B substitutions

To more precisely explore secondary conformations within oxidized Mini-B, FTIR spectroscopy was performed with site-directed, isotope-enriched peptides (50,43,44). Separate peptides were prepared with “clusters” of multiple ¹³C-carbonyl substitutions that were suggested to accurately cover the peptide: Mini-B_{19–34}/SP-B_{19–34} (C¹⁵, 40) at Leu 1, Ala 6, Leu 7, Ile 8, and Ile 11; Mini-B_{8–23}/SP-B_{8–23} (C¹⁵, 3) at Ile 15, Gly 18, Gly 19, and Leu 21; and Mini-B_{8–23}/SP-B_{8–23} (C¹⁵, 3) at Leu 25, Val 26, Leu 29, and Val 30. As the stretching frequencies of the peptide backbone carbonyl groups are sensitive to local conformations, replacement of ¹²C with ¹³C should reduce the stretching frequency of an “isolated” carbonyl oscillator by approxi-

mately 35 per cm (e.g., α -helix band shift from approximately 1657 to 1632 per cm, 33,45). For ¹³C, O substituted into an amide/amide I band with elevated interactions between highly substituted ¹³C-helices in adjacent strands, a maximum frequency shift from approximately 1632 to 1595 per cm should be observed (43,45).

Molecular modeling

The oxidized and reduced Mini-B peptides were each modeled with the CHARMM 22 force field in the molecular 692 environment (see Char of Force, Biomedical Informatics Core Facility). The coordinates for conformations of the oxidized Mini-B in the HFIH solution (i.e., 90% HFIH/90% D₂O), together with a list of restraints, have been deposited in the Protein Data Bank (PDB) under the accession code 1SSZ.

Captive bubble surfactometry

For *in vitro* activity measurements with the captive bubble surfactometer, 0.1 μ mol of protein or peptide was mixed with 35 μ g total phospholipids consisting of 16 mg of DPPC, 10 mg of POPG, 4 mg of POPG, 1 mg of POPG, 1 mg of POPG, and 1 mg of Chol in 1 mL of phosphate-buffered saline (PBS). The surface activity of these surfactant preparations was determined with a captive bubble surfactometer, which has been described in detail elsewhere (47,48). In short, the instrument consists of a sample chamber cut from cylindrical glass (ring of high quality with an inner diameter of 1 cm). A Teflon piston with a tight O-ring seal is fitted into the glass tubing from the top end. A plug of buffered agarose gel is inserted between the piston and solution. The other end is fitted into a plate of stainless steel, which is provided with an inlet port in the center for adding solutions and the air bubble to the chamber. Chamber and piston are vertically mounted within a sturdy rack of steel whose height is regulated by a micrometer gear with minimal redundancy. For usual measurements, the chamber is filled with 0% sucrose (Gibco’s buffer, to which a 3 μ l surfactant is added). The chamber content is stirred with a small magnetic bar, and its temperature is maintained at 37 °C. A small air bubble is introduced from beneath, whose volume and hence surface area can be changed by compression and decompression brought about by changing the height of the rack. The agarose plug’s ionic composition inhibits bubble adhesion to its surface, thus creating an uninterrupted surface area

and a perfect bubble. During the compression and decompression cycles, bubble images are continuously recorded to the hard drive and on a video recorder. Selected single frames are stored in random access memory (RAM) for later image processing and analysis (9). Bubble areas and volumes are calculated by an original algorithm relating bubble height and diameter to areas of revolution, and the bubble surface tension is determined by using the method of Mahlekin and Elford (33).

In vivo measurements with surfactant-deficient rats

For *in vivo* measurements with surfactant deficient rats, oxidized Mini-B surfactant was compared with porcine SP-B_{8–23} surfactant, reduced Mini-B surfactant (SP-B_{8–23}), and surfactant, and mutant Mini-B_{19–34} surfactant, and lipids alone. The surfactants for the *in vivo* experiments had the same lipid and peptide (or protein) compositions as those used in the *in vitro* captive bubble surfactometry determinations. The animal experiments were performed with the approval of the Harborview/VA Research and Education Institute Animal Care and Use Committee (Anesthesia, surgery, lavage, ventilation, and monitoring used in this study are the same as previously described (13,52). Briefly, six groups of eight adult male Sprague-Dawley rats weighing 200–225 g were anesthetized with 35 mg/kg pentobarbital sodium and 80 mg/kg ketamine by intraperitoneal injection, intubated, and ventilated with a rodent ventilator (Harvard Apparatus, Holliston, MA, USA) with 100% oxygen, a tidal volume of 7.5 mL/kg, a positive end-expiratory pressure (PEEP) of 3 cm H₂O, and a rate of 60/min. An arterial line was placed in the abdominal aorta for monitoring blood pressure and blood gases. The rats were paralyzed with 1 mg/kg pancuronium bromide intravenously. Only animals with PaO₂ values of >100 mmHg while ventilated with 100% oxygen were included in the experiments. Airway flow and pressures and tidal volume were monitored continuously with a pneumotachograph connected to the tracheal cannula and a pneumotach system (Fias Rudolph Inc., Kansas City, MO, USA). Dynamic lung compliance was calculated by dividing tidal volume/airway pressure minus PEEP (mL/kg/cmH₂O). The lungs of the rats were lavaged eight times with 8 mL of pre-warmed 0.9% NaCl, and was repeated after 15 min, and, if necessary, after 30 min until the PaO₂ in 100% oxygen reached stable values of <100 torr. After washing in PaO₂, <100 torr,

The rats were treated with 100 mg/kg of one of the six surfactant preparations. The rats were weighed before another 30 min after surfactant instillation. The largest animal model to survive for a period of at least 2 h (12), the rat treated with lipids only were used as negative controls and behave as sham treated animals.

Data are expressed as mean $\pm$ SEM. Between group comparisons were performed by one-way ANOVA, followed by the Student-Newman-Keuls multiple comparison procedure. The *t* test was used for comparisons vs. control values. A *P* value below 0.05 was considered to indicate a significant difference.

Results and Discussion

Structural characterization of Mini-B and related peptides

For native MinB self-films dried from the HEP solution, the predominant peak at 1657 per cm in the conventional ^{13}C -FTIR spectrum (Fig. 1A) is indicative of a nonoligo- α -helix component, while the small shoulder at approximately 1633 per cm suggests minor β -sheet. Because it is not possible from ^{13}C -FTIR spectroscopy to attribute conformational differences to specific residues, local conformational information were probed using isotopically enhanced FTIR spectra of MinB labeled with ^{13}C O groups at neighboring, multiple sites. With MinB-R isotopically enhanced with ^{13}C O labeled at Leu-3, Ala-6, Leu-7, Ile-8, and the 7-helical peak centered at 1657 per cm, and a concurrent increase in a new peak at approximately 1632 per cm (Fig. 1A). The isotopic shift of approximately 35 per cent is due to ^{13}C O labeled Leu-3, Ala-6, Leu-7, and Ile-8 participating in an α helix (6d). The MinB-R isotopically enhanced spectrum (Fig. 1C) also shows a new peak at approximately 1632 per cm and reduced absorption at 1657 per cm, consistent with ^{13}C O labeled Leu-3, Val-26, Leu-39, and Val-40 assuming an α helical conformation. Interestingly, the MinB-R isotopically enhanced (Fig. 1B) presents a distinct pattern with a new peak at approximately 1585 per cm and lowered absorption at approximately 1632 per cm, probably the result of the ^{13}C O labeled residues Ile-16, Val-33, Cys-38, Cys-39, and Leu-23 participating in β -sheet which in turn produces a large shift in the new ^{13}C O peak. This shift is likely due to intermolecular transition dipole coupling interactions between ^{13}C O in adjacent strands when two MinB molecules form an antiparallel β -sheet in the homo-

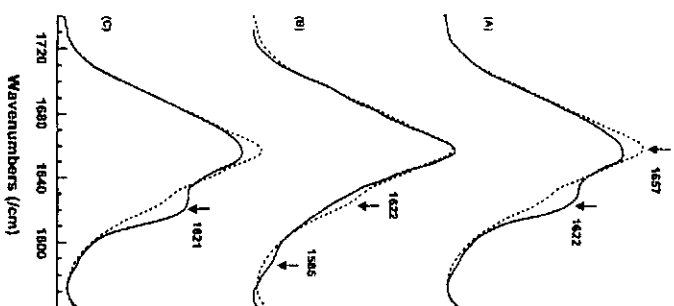


Figure 2. First derivative (solid line) and second derivative (dashed line) of the ^{13}C O-1s intensity of the MIM-8 and a series of oxidized MIM-8 labeled with methoxy- ^{13}C glycidol, for peroxide in 90% isooctane/propylene glycol (MIM-8-90). Peroxide was initially supplied in a 10 wt and dried on the activated solid reference (ATU-Mn-ATU-Mn) at 100°C. The solid line, narrow band (dashed line) (C) (MIM-8-90) and a (dotted line), narrow band (dashed line) (C) (MIM-8-90) were also labeled with, same. MIM-8 (dashed line) See Results and Discussion for spectral assignments of secondary structure.

region (4,7,8). The γ -helices determined here for the N- and C-terminal regions of Mini-B compare favorably with those reported for peptide fragments based on the N-terminal [30,31] or C-terminal [33] domains of SP-B.

Despite the ^{13}C NMR analysis in Fig. 1, the secondary conformation of a small number of residues in MinB were not determined experimentally. Molecular modeling was conducted to provide structural information on these gaps (residues, and also to assess the overall conformational flexibility within MinB. Combining the ^{13}C NMR findings in Fig. 1 with earlier results from residue-specific studies (see above) on N- and C-terminal fragments indicated the following preliminary conformation map for MinB: in a

hydrophobic environment, residues 1–15, 20–21, 25–26, 30–31, 35–36, 39–40, 43–44, 47–48, 51–52, 55–56, 59–60, 63–64, 67–68, 71–72, 75–76, 79–80, 83–84, 87–88, 91–92, 95–96, 99–100, 103–104, 107–108, 111–112, 115–116, 119–120, 123–124, 127–128, 131–132, 135–136, 139–140, 143–144, 147–148, 151–152, 155–156, 159–160, 163–164, 167–168, 171–172, 175–176, 179–180, 183–184, 187–188, 191–192, 195–196, 199–200, 203–204, 207–208, 211–212, 215–216, 219–220, 223–224, 227–228, 231–232, 235–236, 239–240, 243–244, 247–248, 251–252, 255–256, 259–260, 263–264, 267–268, 271–272, 275–276, 279–280, 283–284, 287–288, 291–292, 295–296, 299–300, 303–304, 307–308, 311–312, 315–316, 319–320, 323–324, 327–328, 331–332, 335–336, 339–340, 343–344, 347–348, 351–352, 355–356, 359–360, 363–364, 367–368, 371–372, 375–376, 379–380, 383–384, 387–388, 391–392, 395–396, 399–400, 403–404, 407–408, 411–412, 415–416, 419–420, 423–424, 427–428, 431–432, 435–436, 439–440, 443–444, 447–448, 451–452, 455–456, 459–460, 463–464, 467–468, 471–472, 475–476, 479–480, 483–484, 487–488, 491–492, 495–496, 499–500, 503–504, 507–508, 511–512, 515–516, 519–520, 523–524, 527–528, 531–532, 535–536, 539–540, 543–544, 547–548, 551–552, 555–556, 559–560, 563–564, 567–568, 571–572, 575–576, 579–580, 583–584, 587–588, 591–592, 595–596, 599–600, 603–604, 607–608, 611–612, 615–616, 619–620, 623–624, 627–628, 631–632, 635–636, 639–640, 643–644, 647–648, 651–652, 655–656, 659–660, 663–664, 667–668, 671–672, 675–676, 679–680, 683–684, 687–688, 691–692, 695–696, 699–700, 703–704, 707–708, 711–712, 715–716, 719–720, 723–724, 727–728, 731–732, 735–736, 739–740, 743–744, 747–748, 751–752, 755–756, 759–760, 763–764, 767–768, 771–772, 775–776, 779–780, 783–784, 787–788, 791–792, 795–796, 799–800, 803–804, 807–808, 811–812, 815–816, 819–820, 823–824, 827–828, 831–832, 835–836, 839–840, 843–844, 847–848, 851–852, 855–856, 859–860, 863–864, 867–868, 871–872, 875–876, 879–880, 883–884, 887–888, 891–892, 895–896, 899–900, 903–904, 907–908, 911–912, 915–916, 919–920, 923–924, 927–928, 931–932, 935–936, 939–940, 943–944, 947–948, 951–952, 955–956, 959–960, 963–964, 967–968, 971–972, 975–976, 979–980, 983–984, 987–988, 991–992, 995–996, 999–1000, 1003–1004, 1007–1008, 1011–1012, 1015–1016, 1019–1020, 1023–1024, 1027–1028, 1031–1032, 1035–1036, 1039–1040, 1043–1044, 1047–1048, 1051–1052, 1055–1056, 1059–1060, 1063–1064, 1067–1068, 1071–1072, 1075–1076, 1079–1080, 1083–1084, 1087–1088, 1091–1092, 1095–1096, 1099–1100, 1103–1104, 1107–1108, 1111–1112, 1115–1116, 1119–1120, 1123–1124, 1127–1128, 1131–1132, 1135–1136, 1139–1140, 1143–1144, 1147–1148, 1151–1152, 1155–1156, 1159–1160, 1163–1164, 1167–1168, 1171–1172, 1175–1176, 1179–1180, 1183–1184, 1187–1188, 1191–1192, 1195–1196, 1199–1200, 1203–1204, 1207–1208, 1211–1212, 1215–1216, 1219–1220, 1223–1224, 1227–1228, 1231–1232, 1235–1236, 1239–1240, 1243–1244, 1247–1248, 1251–1252, 1255–1256, 1259–1260, 1263–1264, 1267–1268, 1271–1272, 1275–1276, 1279–1280, 1283–1284, 1287–1288, 1291–1292, 1295–1296, 1299–1300, 1303–1304, 1307–1308, 1311–1312, 1315–1316, 1319–1320, 1323–1324, 1327–1328, 1331–1332, 1335–1336, 1339–1340, 1343–1344, 1347–1348, 1351–1352, 1355–1356, 1359–1360, 1363–1364, 1367–1368, 1371–1372, 1375–1376, 1379–1380, 1383–1384, 1387–1388, 1391–1392, 1395–1396, 1399–1400, 1403–1404, 1407–1408, 1411–1412, 1415–1416, 1419–1420, 1423–1424, 1427–1428, 1431–1432, 1435–1436, 1439–1440, 1443–1444, 1447–1448, 1451–1452, 1455–1456, 1459–1460, 1463–1464, 1467–1468, 1471–1472, 1475–1476, 1479–1480, 1483–1484, 1487–1488, 1491–1492, 1495–1496, 1499–1500, 1503–1504, 1507–1508, 1511–1512, 1515–1516, 1519–1520, 1523–1524, 1527–1528, 1531–1532, 1535–1536, 1539–1540, 1543–1544, 1547–1548, 1551–1552, 1555–1556, 1559–1560, 1563–1564, 1567–1568, 1571–1572, 1575–1576, 1579–1580, 1583–1584, 1587–1588, 1591–1592, 1595–1596, 1599–1600, 1603–1604, 1607–1608, 1611–1612, 1615–1616, 1619–1620, 1623–1624, 1627–1628, 1631–1632, 1635–1636, 1639–1640, 1643–1644, 1647–1648, 1651–1652, 1655–1656, 1659–1660, 1663–1664, 1667–1668, 1671–1672, 1675–1676, 1679–1680, 1683–1684, 1687–1688, 1691–1692, 1695–1696,

Previous theoretical and physical experiments on peptides based on the N- and C-terminal domains suggest that MimB partitions into the polar headgroup region of lipids. Electron spin resonance (ESR) and fluorescence spectroscopy of SP-B₁₋₂₂ indicate a surface location in lipid bilayers, while the N-terminal helix (i.e., MimB residues 1–15) [38] and the two-dimensional nuclear magnetic resonance (2D-NMR) of the C-terminal SP-B₁₅₋₂₂ in SDS micelles showed that its helix (i.e., MimB residues 24–44) was restricted to the water interface [33]. These results agree with predictions using hydrophobic moment plots that the N- and C-terminal helical domains of SP-B are surface membrane-anchoring (53). Because the disulfide-linked N- and C helical

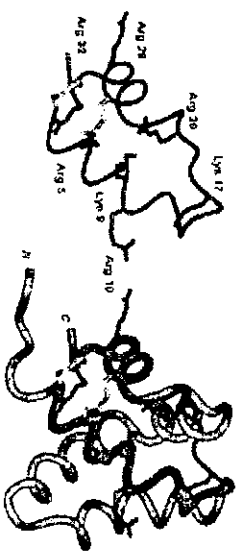


Figure 2. (A) Labelled backbone representation of the MinI construct (a recombinant subunit produced in the overexpression of 90% of the full-length autophagy gene, *Atg18*, in *Arabidopsis thaliana*). The ribbon representation is based on a single conformation of Protein Data Bank (PDB) entry 1G5Z. A helical domain, well identified in all of MinI constructs (1-181 terminal helix) and 1-197 (terminal helix), where the secondary loop region connecting these helical domains is shown in blue. The side groups of the positively charged Arg and Lys residues are shown in purple. Disulfide linkage between cysteines 250 and 256 (Cys254 and Cys256) are yellow. (B) Labelled backbone representation of the MinI recombinant (150-mer) structure and cysteines shown as sticks overlaid on the corresponding, red and teal, terminal regions of the full-length 354-mer protein (see text for details). Residues 150-197 are shown in teal overlaid on the corresponding, red, while the corresponding C-terminal 150-mer is labelled as C.

these domains are surface-seeking helices (data not shown). domains probably assume compact structures both in native SPr-B and in lipid bilayers (32). The association of native SPr-B in lipid bilayers (33) may be due to the resistance of these charged amphipathic domains to deep penetration. Interestingly, analogous "anti-B" configurations have been reported for the N and C terminal domains of other proteins: e.g., NK-lysin, sapsin B, and C, that share the sapsin fold (16,17,54), and may also participate in the association of these proteins with membrane lipid bilayers (16). Hydrophobic moment analysis of the N and C termini regions for NK-lysin, sapsin B, and C confirms that these domains are surface-seeking helices (data not shown).

The overall secondary conformations of several Mini-B variants and fragments were analyzed using conventional ^{13}C -FTIR spectroscopy. The FTIR spectrum of reduced Mini-B-Minitri-11c, no intrachain disulfide linkages) in the HEPES buffer was nearly identical to that of oxidized Mini-B-solvent, suggesting that the oxidized and reduced forms share similar secondary structures. Unlike the conjugated oxidized Mini-Bs that were found to be conformationally inflexible due to two disulfide bridges (Fig. 2A), dynamically flexible due to two disulfide bridges (Fig. 2A), dynamic energy minimizations of reduced Mini-B show that the N- and C-terminal α -helices "flail" about the central coiled Mini-B triifluc, an oxidized Mini-B variant with positively charged residues replaced by threonines in the HEPES solvent also showed a predominant α -helical component (Fig. 3B). However, the small shift in the Mini-B triifluc spectrum to lower wavenumbers indicate that the introduction of lower than three times probably enhanced randomly-coiled structures by slightly flaying the α -helices of the N- and C-terminal regions. Interestingly, the FTIR spectrum of the SP-B₁₋₂₂

and SP-B_{1-27} fragments in HEP solvent [pH 7.4] closely mimicked that of the isolated Amino spectrum, even though the fragments were not covalently linked. The high-resolution mass spectrometry results of the SP-B_{1-27} and SP-B_{1-28} peptides found here for the mixture of SP-B_{1-27} and SP-B_{1-28} in the HEP solvent are consistent with previous studies [10]. The m/z values of the SP-B_{1-27} and SP-B_{1-28} peptides of SP-B in combination minus 1 (m/z 3,351

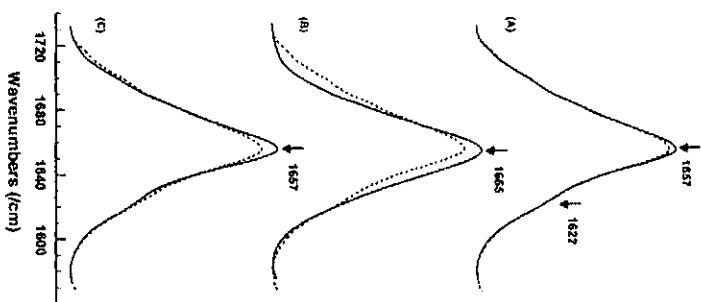


Figure 3. Conventional ^{13}C NMR spectrum (containing infrared) spectra of the condensed Mon II peptide and Mon III; carboxyls and amines are in D_2O . Inhomogeneous signals (labeled H_2O , D_2O) appear with intensity in gD_2O and dried on the averaged total; otherwise, in H_2O phase. (a) Inhomogeneous in H_2O phase; (b) isolated Mon II peptide; (c) isolated Mon III peptide; (d) condensed Mon II peptide; (e) condensed Mon III peptide. (f) Mixture of SP II, III, and SP IV, V. (g) Peptide fragments (isolated from condensed Mon II (labeled line)). The split, α and β - NH_2 peptides are marked red according to the 29 sequence of the split; γ - NH_2 is shown, and correspond to residues 1–15 and 20–34, respectively. The α and β are marked by experimental procedures; γ is marked by discussion for special assignments of secondary structure.

Captive bubble surfactometry of SP-B, oxidized Mini-B, and related peptides

As Mini B assumes the 3-D organization of the N and C terminal regions in the SP-B protein (Fig. 2B), Mini-B should also reproduce key *in vitro* and *in vivo* activities of native SP-B. If this structure is important for SP-B function, as has been noted [56], several criteria must be met for a surfactant preparation to be functional, including rapid adsorption to the air-water interface, attainment of near-zero surface tension upon film compression, and fast and efficient respreading of the surfactant on expansion of the interface. Using captive bubble surfactometry [49], *in vitro* film formation and dynamics were studied for surfactant preparations containing SP-B, oxidized Mini B, or released peptides. The adsorption of surfactant preparation in the subphase to a clean interface of the air bubble in the bubble chamber is readily determined from changes in the bubble shape. Lipid surfactants, either with or without protein, were injected into the chamber. A bubble was then created and adsorption was allowed for 5 min. The chamber was then sealed and the initial bubble volume (approximately 600 μm^3) measured. The surface tension shows at this time are reported last cycle (9 in Fig. 4). Except for the mutant Mini-B(13-41) preparation, surfactants prepared for the mutant Mini-B(13-41) and (SP-B)₁₋₂₄ (SP-B₁₋₂₄) each exhibit similar activity compared with that of the native sample (approximately 35–50 mN/m vs. approximately 5.7 mN/m). However, these values for the Mini-B peptides are significantly higher than that for native SP-B (approximately 3.5 mN/m, Fig. 4), indicating that these peptides only partly reproduce the surface tension-lowering properties of the native protein at cycle 9. A similar partial mimicking of the initial surface activity of native SP-B was observed in captive bubble surfactometry of peptides based solely on the N-terminal domain of SP-B (Fig. 4), and here Veldhuizen *et al.* [50] suggested that the N-terminal peptides are more resistant than the full length SP-B in preventing an existing, relatively tight-packed lipid layer at cycle 9. Interestingly, the mutant Mini-B(13-41) preparation showed a very high surface tension approaching that of the charged lipid sample (Fig. 4), suggesting that positive charge reduces or a necessary requirement for *in vitro* surfactant activity.

The maximum and minimum surface tensions ($\gamma_{\max}$ and $\gamma_{\min}$) during cycling with these surfactant mixtures are illustrated in Fig. 4. The nontreated and reduced MonomK preparations have increased activity compared with the control lipid samples, with both cycle $\gamma_{\max}$ -values of

to mN/m for both oxidized and reduced mmt-B (see the respective T_{max} values are approximately 13 and 17 mN/m). Interestingly, the both cycle T_{max} for native SP-B (approximately 6 mN/m) agrees to within experimental error with the corresponding T_{max} values for the oxidized and reduced Mmt-B preparations (Fig. 4), while the ranking for the both cycle T_{max} values is SP-B (approximately 33 mN/m) $\leq$ oxidized Mmt-B (approximately 41 mN/m) $\leq$ reduced Mmt-B (approximately 41 mN/m) (Fig. 4). These data indicate that oxidized Mmt-B accurately mimics the *in vitro* cycling surface activity of the full-length SP-B. While the reduced Mmt-B also promotes near zero surface tension upon film compression (Fig. 4), its relatively high both cycle T_{max} value suggests that disulfide linkages are necessary for Mmt-B to express optimal *in vitro* surfactant activity. It should be also noted that the physical continuity and positive-charge distribution of the 3 helical N- and C-terminal SP-B domains are critical for *in vitro* surface activity, as [SP-B₁₋₃]-SP-B₁₋₃ and mutant Mmt-B(Mt) each show high surface tensions upon expansion and compression (Fig. 4).

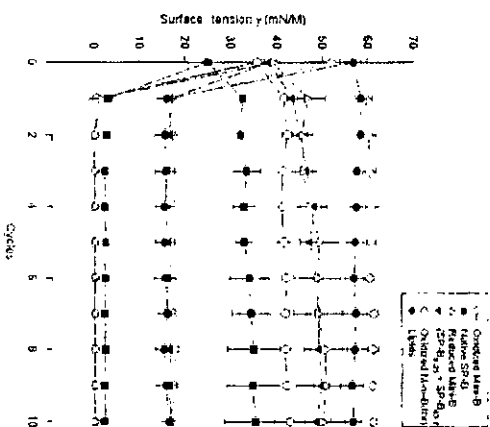


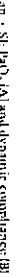
Figure 3 presents the average surface profiles of the six satisfaction (post-experiment) used in the experiments. About 1 µl of each of the surface preparations was injected into the syringe and allowed to spread to the interface for 5 min. Maximum and minimum surface values were determined by using a 1 × 0.41 mm pin for the various surface preparations. The average values are shown in Table 1. The average values of the surface profiles of polished films are indicated. Surface profile values shown are the average of at least four separate experiments. I-SMNT

Rats demonstrated a rapid recovery of oxygenation (PaO_2) and dynamic compliance (tidal volume/kg body weight) after resuscitation with oxidized Mini-B, native SP-B, reduced Mini-B, and (SP-B)₁₋₁₁ - SP-B₁₂₋₂₈ but not with mutant Mini-B(181) surfactant or lipids alone (Fig. 3). The average PaO_2 values following lung lavage were 14% of the pre-lavage values and increased after treatment with oxidized Mini-B, native SP-B, reduced Mini-B, (SP-B)₁₋₁₁ - SP-B₁₂₋₂₈, mutant Mini-B(181), and lipids alone to 99%, 87%, 71%, 40%, 22% and 20% of the pre-lavage values, respectively. Post-lavage dynamic compliance dropped to 63% of the pre-lavage values and increased after treatment with oxidized Mini-B, native SP-B, reduced Mini-B, (SP-B)₁₋₁₁ - SP-B₁₂₋₂₈, mutant Mini-B(181), and lipids alone to 85%, 79%, 66%, 73%, 70%, and 69% of the pre-lavage values, respectively. PaO_2 values for oxidized Mini-B and native SP-B were significantly higher than those of the other surfactant preparations or lipids alone when tested with one-way ANOVA ($P < 0.001$ and $P < 0.05$, respectively).

Dynamic compliance values for oxidized Mini-B surfactant and native SP-B surfactant exceeded those of the other surfactant preparations and lipids alone, but only the differences with lipids alone were statistically significant ($P < 0.001$ and $P < 0.05$, respectively). The differences between oxidized Mini-B and native SP-B surfactant were not statistically significant.

Conclusions

Our results show that Mimn-B, a synthetic construct that includes the α -helical N- and C-terminal domains and closely mimics important components of the septin-folding pattern of SP-B, also reproduces key *in vitro* and *in vivo* functions of full-length SP-B. Reducing the positive charge of Mimn-B diminishes the ability of this peptide to promote faster surface tension on film compression and reproducibly resperdiging, both of which appear to be important for proper *in vivo* performance. Similar to native SP-B, isolated Mimn-B probably interacts through its cationic residues (i.e., lysine, arginine) with anionic lipids such as phosphatidylglycerol (15), thereby counteracting the driving force for lipid squeeze-out from the surface film. This relative *in vitro* surfactant activities of Mimn-B and its variants, if up of cardiac surfactant with the relative oxygenation and lung compliance in surfactant-deficient animals.



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both Moa-H and native, full-length SP-R (Figs 4 and 5) support the hypothesis that surfactant functions of SP-R are at least partially localized to a disk-like structure containing the disulfide-linked, charged amphipathic N- and C-terminal domains (Fig. 1a).

Both *in vitro* and *in vivo* activities [Figs. 4 and 5] showed that the function of Mtm-B (and hence native Slp-B) is dependent on its 3D structure and charge distribution. Further reduction of the disulfide linkages of Mtm-B or complete disconnection of the N- and C-terminal helical domains yielded abnormal compression-expansion curves (Fig. 4) and reduced oxygenation in surfactant-deficient rats (Fig. 5). Consequently, our results suggest that the sapro- and folding pattern, observed for both Mtm-B and Slp-B (Fig. 2), participates in the optimal expression of surfactant activity.

This surface–function model explains why peptides based on only the N- and C-terminal domains are not able to fully reproduce the surface activities of full-length SPI-B, as well as the topographical organization (i.e. sapsin fold) of these disulfide-linked N- and C-terminal regions is only partially mimicked. Of more general interest is the finding that comparable Mini B configurations have been observed in the N- and C-terminal domains of other sapsin proteasins (e.g. NfK1 β sasin, sapsin-A and C) insert into lipid bilayers (16, 20), our present findings with the Mini B action proteasins of other sapsin proteins may be generally due to respective 'Mini-B' domains.

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