Differential Partitioning of Pulmonary Surfactant Protein SP-A into Regions of Monolayers of Dipalmitoylphosphatidylcholine and Dipalmitoylphosphatidylcholine/Dipalmitoylphosphatidylglycerol

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ABSTRACT The interaction of the pulmonary surfactant protein SP-A fluorescently labeled with Texas Red (TR-SP-A) with monolayers of dipalmitoylphosphatidylcholine (DPPC) and DPPC/dipalmitoylphosphatidylglycerol 7:3 w/w has been investigated. The monolayers were spread on aqueous subphases containing TR-SP-A. TR-SP-A interacted with the monolayers of DPPC to accumulate at the boundary regions between liquid condensed (LC) and liquid expanded (LE) phases. Some TR-SP-A appeared in the LE phase but not in the LC phase. At intermediate surface pressures (10–20 mN/m), the protein caused the occurrence of more, smaller condensed domains, and it appeared to be excluded from the monolayers at surface pressure in the range of 30–40 mN/m. TR-SP-A interaction with DPPC/dipalmitoylphosphatidylglycerol monolayers was different. The protein did not appear in either LE or LC but only in large aggregates at the LC-LE boundary regions, a distribution visually similar to that of fluorescently labeled concanavalin A adsorbed onto monolayers of DPPC. The observations are consistent with a selectivity of interaction of SP-A with DPPC and for its accumulation in boundaries between LC and LE phase.

INTRODUCTION

Surfactant protein A (SP-A) is the major protein associated with pulmonary surfactant, a complex lipid–protein material that covers the alveolar surface, reduces the surface tension of the air–liquid interface, and facilitates respiratory mechanics. It is widely assumed that an interfacial phospholipid monolayer enriched in dipalmitoylphosphatidylcholine (DPPC) is mainly responsible for the tensoactive properties of lung surfactant and that phosphatidylglycerol and surfactant-associated proteins SP-A, SP-B, and SP-C are also required for the formation and proper dynamics of the surfactant monolayer in the airways (Keough, 1992; Kuroki and Voelker, 1994; Johansson et al., 1994).

SP-A is a hydrophilic glycoprotein with a monomeric molecular mass of 30–40 kDa (Hawgood and Shiffer, 1991). Its N-terminal moiety has a collagen-like sequence, whereas the C-terminal portion is like a C-type lectin with binding sites for carbohydrates and Ca$^{2+}$ (White et al., 1985; Drickamer, 1988). The native form of the protein as isolated from the alveolar spaces is assembled into a complex oligomer in which SP-A chains are organized in trimers through collagen-like triple-helices, and six trimers form a bouquet-like arrangement similar to that of the protein C1q from the complement system (Voss et al., 1988).

Studies in vitro have shown that SP-A interacts with surfactant phospholipids and glycosphingolipids both in bilayers and immobilized on silica gel plates with a selectivity for interaction with DPPC and galactosylceramide (King et al., 1983; Kuroki and Akino, 1991; Kuroki et al., 1992; Childs et al., 1992; Casals et al., 1993). SP-A induces phospholipid vesicle aggregation in the presence of Ca$^{2+}$ (King et al., 1983; Hawgood et al., 1985; Ruano et al., 1996). These lipid-binding activities mediate the participation of SP-A in processes of surfactant metabolism in the alveolar spaces. SP-A, for instance, is required for the generation in vitro of tubular myelin (Suzuki et al., 1989; Williams et al., 1991), a structure that has been associated with highly tensoactive surfactant preparations. SP-A also participates in the regulation of surfactant secretion into and clearance from the alveolar spaces (Dobbs et al., 1987; Bates et al., 1994; Wright and Youmans, 1995) and in several activities associated with alveolar defense against pathogens (Van Golde, 1995). In addition, surfactant containing SP-A is more resistant than surfactant without SP-A to the inhibition of surfactant activity induced by plasma proteins such as fibrinogen or albumin (Cockshutt et al., 1990; Strayer et al., 1996).

Although SP-A by itself is poor at accelerating the transfer of surfactant phospholipids to the air–liquid interface, it augments rapid formation of phospholipid surface films in cooperation with the hydrophobic surfactant proteins SP-B and SP-C (Hawgood et al., 1987; Pison et al., 1990; Schurch et al., 1992). SP-A seems also to stabilize the surfactant monolayer at low surface pressures and enhance elimination of non-DPPC lipids during compression (Schurch et al., 1992). Korfhagen and co-workers (1996) have shown that SP-A knock-out mice can breathe properly and present unaltered lung morphology and function, suggesting that SP-A is not essential for respiratory function at least in the perinatal stage. However, in that study the minimal surface
tension of monolayers formed from material extracted from SP-A-defective lungs was higher than that of surfactant from control animals. Taneva et al. (1995) have shown that at pH 7.4 in the absence of Ca$^{2+}$, SP-A interacts and perturbs pure DPPC monolayers, but it shows complete immiscibility with negatively charged monolayers composed of DPPG or a combination of DPPC and DPPG.

In the present study we investigated the association of SP-A from the hypophase with interfacial spread monolayers of DPPC or DPPC/DPPG by epifluorescence microscopy (Pérez-Gil et al., 1992a; Nag and Keough, 1993; Nag et al., 1996b). The use of fluorescently labeled proteins allows for the analysis of interactions of a given protein with domains or regions of the monolayer (Grainger et al., 1990; Maloney et al., 1995; Nag et al., 1996a).

**EXPERIMENTAL PROCEDURES**

**Materials**

The phospholipids, 1,2-dipalmitoylphosphatidylcholine and 1,2-dipalmitoylphosphatidylglycerol (DPPG) were purchased from Sigma Chemical Co. (St. Louis, MO). The fluorescent lipid probe 1-palmitoyl-1-[12-[(7-nitro-2-1,3-benzoxadizole-1-yl)amino]dodecanoyl]phosphatidylcholine (NBD-PC) was from Avanti Polar Lipids (Birmingham, AL). The fluorescent-labeling chemical sulforhodamine 101 sulfonyl chloride, Texas Red$^\text{TM}$ (TR), was obtained from Molecular Probes Inc. (Eugene, OR), as was Texas Red-labeled concanavalin A (TR-conA). Chloroform and methanol were HPLC grade solvents from Fisher Scientific Co. (Ottawa, ON), and all other reagents were analytical grade chemicals from Merck (Darmstadt, Germany).

**Isolation and Labeling of SP-A**

Surfactant protein A was purified from pulmonary surfactant prepared from porcine bronchoalveolar lavage as previously described (Casals et al., 1989) by sequential butanol and octylglucoside extractions (Casals et al., 1993) and stored at −20°C in solutions in 5 mM Tris-HCl buffer, pH 7.4. Purity of the protein preparations was routinely checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions followed by Coomassie Blue staining. SP-A was quantitated by amino acid analyzer. 

Fluorescently labeled SP-A was prepared as follows. Solutions of SP-A in 5 mM Tris-HCl buffer containing 300–500 µg of protein were adjusted to pH 8.0 by addition of 50 mM Tris, pH 8.3. The labeling reaction was started by the addition of 1 mM TR in methanol to a final SP-A/TR ratio of 5–6:1 (mol/mol). The mixture was incubated for 90 min in darkness at room temperature and then exhaustively dialyzed against 5 mM Tris-HCl, pH 7.4, to remove unreacted fluorescent reagent. Activity of labeled TR-SP-A compared with that of native protein was assayed by testing its ability to induce aggregation of DPPC vesicles in the presence of Ca$^{2+}$ at 37°C as described elsewhere (Ruano et al., 1996).

**Epifluorescence Experiments**

Surface pressure-area measurements and microscopic observations of phospholipid monolayers were performed on an epifluorescence microscope surface balance, the construction and operation of which have been described elsewhere (Nag et al., 1990; 1991). To form monolayers, the lipids, DPPC or the mixture DPPC/DPPG (7:3, w/w), were mixed in chloroform/methanol (3:1, vol/vol), and 1 mol % of NBD-PC was included. Monolayers were spread by depositing very small aliquots of the chloroform/methanol solutions on a subphase of 5 mM Tris-HCl, pH 7.4, and 150 mM NaCl with or without SP-A or TR-SP-A. All subphases were prepared with double distilled water, the second distillation being from dilute potassium permanganate. After spreading of a monolayer, the organic solvent was allowed to evaporate for 5 min, and, in order to facilitate SP-A adsorption to the air-liquid interface, the monolayer was compressed rapidly (707 mm$^2$/s) to a surface pressure of 10 mN/m and then expanded again to 0 mN/m. After a 1-h period, which would have allowed for penetration of the protein into the gas or gas-liquid expanded coexistence phases (e.g., Maloney et al., 1995), the monolayer was compressed at a slow speed (20 mm$^2$/s) or an initial rate of 0.13 Å$^2$/molecule/s at 23 ± 1°C. At selected surface pressures, a video recording was made for a 1-min period of both NBD and TR fluorescence by switching fluorescence filter combinations. The images obtained were analyzed with digital image processing using JAVA 1.3 software (Jandel Scientific, San Rafael, CA) as discussed elsewhere (Nag et al., 1991; Pérez-Gil et al., 1992a).

The video images were obtained with a CCD camera that records in black and white. The images presented in the figures have been false-colored to display them as they appear to the eye in the microscope.

**RESULTS**

Fig. 1 shows Ca$^{2+}$-dependent DPPC vesicle aggregation induced by native SP-A and TR-SP-A prepared under two different conditions. Incubation of SP-A with TR under mild conditions, 90 min at room temperature and pH 8.0, 1°C. 

![FIGURE 1](image-url)
yielded a labeled protein with vesicle-aggregating activity similar to that of native SP-A. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, this modified protein showed a main fluorescent band centered at 35 kDa similar to that of native SP-A (data not shown). Incubation of SP-A with TR for 17 h at 4°C produced a modified protein with higher fluorescence as observed in electrophoresis gels but having a lower ability to induce aggregation of DPPC vesicles (Fig. 1, closed triangles). This decrease in activity is possibly because of structural alterations in SP-A caused by extensive addition of probe molecules to amine groups of the protein. The milder conditions were selected to prepare fluorescent TR-SP-A for additional experiments. After exhaustive dialysis, spectroscopic measurements of the TR-SP-A samples prepared in this way yielded an estimate of incorporation of around 0.4 moles of probe per mole of protein monomer.

Fig. 2 shows the surface pressure — area per molecule (II-A) isotherms of DPPC monolayers spread on subphases containing different concentrations of TR-SP-A. The isotherms showed that increasing concentrations of the protein in the subphase caused a progressive expansion of the interfacial DPPC film, suggesting that the protein is occupying some space in the interface, or, at least it is interacting with the phospholipid monolayer sufficiently to perturb the usual lipid packing. All of the monolayers showed LE (liquid expanded) to LC (liquid condensed) transitions as deduced from the plateau regions of the isotherms at surface pressures in the range of 7–9 mN/m. The effect of TR-SP-A on the II-A isotherms of DPPC was qualitatively and quantitatively similar to the effect of native SP-A (data not shown) and analogous to the effect of native SP-A in DPPC/SP-A monolayers spread from solvents (Taneva et al., 1995).

Microscopic observations of monolayers of DPPC containing 1 mol % NBD-PC, in the absence and in the presence of TR-SP-A, showed typical LE-LC coexistence regions consisting of dark LC regions excluding the fluorescent probe and bright green areas of LE phase similar to those previously observed in lipid and lipid/protein monolayers (Nag et al., 1991; Pérez-Gil et al., 1992a; Nag et al., 1996a; 1996b). The transition of a monolayer from LE to LC phase upon compression can be quantitatively de-
scribed by parameters such as the size and number of condensed domains and the percentage of total condensed phase as a function of the compression pressure (Peschke and Möhwald, 1987; Nag et al., 1991; Pérez-Gil et al., 1992a; Nag and Keough, 1993). Such an analysis is shown in Fig. 2 B for monolayers formed on subphases in the absence and in the presence of 0.06, 0.13, and 0.26 μg/ml of TR-SP-A. The data indicate that over the range of π of 5–30 mN/m, TR-SP-A 1) increases the number of condensed domains per frame, 2) decreases the average area of condensed domains, and 3) decreases the percentage of total condensed phase at lower surface pressures. Above surface pressures of about 35 mN/m, there were no differences in the total amount of condensed phase in monolayers formed in the absence or presence of TR-SP-A. This is consistent with the observation that SP-A is substantially excluded from DPPC monolayers at that surface pressure (Taneva et al., 1995).

Fig. 3 shows images obtained from a monolayer of DPPC containing 1 mol % of NBD-PC formed on a subphase containing 0.06 μg/ml TR-SP-A for fluorescence selectively coming from either NBD-PC or TR-SP-A. NBD fluorescence showed phospholipid LC domains with shapes similar to, but somewhat more irregular than, those of the elliptical kidney-bean-shaped condensed domains of pure DPPC (Nag et al., 1991; Pérez-Gil et al., 1992a). It is noted that the fluorescence of TR-SP-A was not observed in the monolayers at very low pressures (below 3–4 mN/m) and only was seen when LC domains began to appear. Changes in the numbers, shapes, and sizes of LC domains have been

![Image](https://example.com/image.png)

FIGURE 3  (a) Typical images obtained from a DPPC monolayer containing 1 mol % NBD-PC spread on a subphase containing 0.06 μg/ml of TR-SP-A at the surface pressures indicated. Images were recorded through filters selecting fluorescence coming either from NBD-PC (emission centered at 520 nm) or TR-SPA (emission centered at 590 nm). (b) Typical image of a single condensed domain at a surface pressure of 12 mN/m seen through the TR filter. The white line indicates the trajectory that TR fluorescence intensity was quantitatively analyzed in graph c. (c) Relative fluorescence intensity was evaluated in pixels along the line marked in image b and plotted against the distance in μm from the starting point in the dark domain. (d) The average fluorescence intensities of the regions defined in graph c plotted against surface pressure. Data are presented as $\bar{x} \pm SD$ for $n = 10$ images. Error bars not shown are within the symbol sizes.
described as one of the consequences of lipid/protein inter-
actions in the monolayer (Pérez-Gil et al., 1992a; Nag et al.,
1996a; 1996b). At surface pressures between 5 and 30 
mN/m, the fluorescence from TR-SP-A showed that it as-
sociated with the fluid phase and the condensed/fluid
boundaries of the monolayer. Three different regions in the
monolayer can be defined according to the average intensity
of fluorescence coming from TR-SP-A (Fig. 3, A to C). LC
regions essentially lack any TR-SP-A fluorescence, sug-
gesting either that SP-A does not associate directly with
DPPC in LC phase or that TR-SP-A fluorescence is not
accessible in the microscope when the protein is associated
with the tightly packed headgroups of DPPC condensed
domains. LE regions (those showing NBD fluorescence)
had homogeneous TR-SP-A fluorescence intensity, suggest-
ing a regular distribution of SP-A in the fluid areas of the
monolayer. Finally, an intense ring of TR fluorescence
surrounded the DPPC condensed domains in the boundaries
between LC and LE regions. As the pressures increased, the
TR fluorescence that accumulated at the boundaries de-
creased (Fig. 3 A). Quantitative analysis of the TR fluores-
cence intensity of the different regions of the monolayer
(Fig. 3 D) suggests that the average TR-SP-A fluorescence
of the LC-LE boundaries converged to the fluorescence
intensity of the LE areas at surface pressures above 20 
mN/m. At this pressure, however, the total amount of LE
phase in DPPC monolayers is relatively small (Nag et al.,
1991; Pérez-Gil et al., 1992a).

The effect of TR-SP-A on DPPC/DPPG (7:3, w/w)
monolayers is shown in Fig. 4. Fig. 4 A shows typical II-A
isotherms of monolayers of DPPC/DPPG (7:3, w/w) con-
taining 1 mol % NBD-PC spread on subphases containing 0,
0.06, 0.13, and 0.26 μg/ml of TR-SP-A. These isotherms
are somewhat similar to those obtained for pure DPPC and
DPPC/TR-SP-A systems with LE-LC plateaus also in the
range 6–9 mN/m. The fluorescence coming from the lipid
probe, however, showed a different effect of SP-A on
DPPC/DPPG compared with the effect of SP-A on DPPC
monolayers. TR-SP-A did not have any significant effect on
the number and size of condensed domains or the percent-
age of total condensed phase of DPPC/DPPG monolayers at
any surface pressure. The distribution of TR-SP-A in these
phosphatidylglycerol-containing monolayers was also dif-
ferent from that observed for the protein in monolayers of

**FIGURE 4**  (A) Typical II-A isotherms of DPPC/DPPG (7:3, w/w) monolayers containing 1 mol % NBD-PC spread on subphases 150 mM NaCl, 5 mM
Tris-HCl, pH 7.4, containing the indicated amounts of TR-SP-A. Number (B) and average area (C) of the condensed domains and the percent of total
condensed area (D) are plotted against surface pressure (P) for various protein concentrations in the subphase. Values are x ± SD for n = 10 images. Error
bars not shown are within the symbol sizes.
DPPC alone. In DPPC/DPPG monolayers, TR-SP-A was located in discrete aggregates of sizes in the range of 5–10 μm at nearly all surface pressures of the isotherm (Fig. 5). Almost no TR fluorescence could be detected in either LC domains or LE regions. The fluorescent protein aggregates were also preferentially located at the boundaries between LC and LE regions as deduced from the comparison of images taken from both NBD and TR filters (Fig. 5).

To determine if the distribution of SP-A in phospholipid monolayers is specific for this protein, we studied the distribution of another fluorescently labeled glycoprotein, TR-concanavalin A, in DPPC monolayers. Concanavalin A, a tetrameric glycoprotein with a molecular mass of 104 kDa has lectin activity as does SP-A, but to our knowledge it is not a lipid-binding protein. Fig. 6 shows typical images obtained from DPPC monolayers spread on a subphase containing TR-conA at 0.26 μg/ml. Protein fluorescence from these monolayers indicated that the protein formed aggregates similar in size and shape to those formed by SP-A in DPPC/DPPG monolayers. Homogeneously distributed protein fluorescence was not detected in either LC or LE regions. The fluorescent protein patches also showed some preference for distribution next to the borders of the LC domains, although this preference seemed to be less striking than in the case of SP-A in DPPC/DPPG monolayers.

**DISCUSSION**

These experiments demonstrate that SP-A in the hypophase can interact and associate with interfacial monolayers formed with the main surfactant phospholipid DPPC. The observed association of SP-A with the monolayer could be a consequence of the intrinsic affinity of the protein to interact with lipids, especially DPPC, as previously demonstrated with phospholipid vesicles (King et al., 1983; Casals et al., 1993) or immobilized lipids on silica gel plates or on plastic (Kuroki and Akino, 1991; Kuroki et al., 1992; Childs et al., 1992).

The presence of SP-A in monolayers of DPPC produced similar perturbations in the condensation of the monolayer under compression as those described for other proteins, for instance SP-C (Pérez-Gil et al., 1992a). The magnitude of the perturbation caused by SP-A appears relatively small on the basis of mass of protein but could be significant on the basis of the molar amount of protein associated with the monolayer in comparison with a smaller protein such as SP-C (Pérez-Gil et al., 1992a). As discussed previously (Pérez-Gil et al., 1992a), the influence of the protein to produce more smaller-condensed domains than are seen in the pure lipid likely reflects a compromise of forces between the perturbing influence of protein in the monolayers.
resisting condensation and the increasing applied pressure that promotes that condensation. The perturbing influence of SP-A is seen only in the range of $p$ of about 5–30 mN/m (Fig. 2). Above that pressure, SP-A is primarily squeezed-out of DPPC monolayers (Taneva et al., 1995) or into small residual SP-A aggregates in or near the surface (Fig. 2). It is possible that all the proteins with ability to interact with phospholipids in monolayers will show similar effects on the condensation. In terms of its preferential accumulation at the boundaries of LC domains, however, SP-A is dramatically different from SP-C, for example, which shows uniform distribution in the LE phase.

SP-A is a water-soluble protein that can interact with selected lipids. Some of the work on the visual properties of interactions between water soluble protein and monolayer films has been summarized in Ahlers et al. (1990) and Mohwald (1990). For example, a fluorescently labeled analog of pancreatic phospholipase A$_2$ showed selective association with solid phase domains in DPPC monolayers (Taneva et al., 1995) or into small residual SP-A aggregates in or near the surface (Fig. 2). It is possible that all the proteins with ability to interact with phospholipids in monolayers will show similar effects on the condensation. In terms of its preferential accumulation at the boundaries of LC domains, however, SP-A is dramatically different from SP-C, for example, which shows uniform distribution in the LE phase.

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The effect of SP-A in DPPC/DPPG monolayers is different compared with that seen in DPPC monolayers. The protein showed almost no effect on the condensation parameters of DPPC/DPPG monolayers. Homogeneous TR-SP-A fluorescence was not detectable in LE regions, but discrete patches close to the LE-LC boundaries were observed. This would be consistent with very little or no interaction of SP-A with negatively-charged monolayers. Similar differential behavior of SP-A was found in solvent-spread monolayers of DPPC and DPPC/DPPG (Taneva et al., 1995), and SP-A interacts more strongly with DPPC than with DPPG or DPPC/DPPG bilayers (Casals et al., 1993). Interaction of SP-A with negatively charged phospholipid bilayers was completely abrogated at low ionic strength (Casals et al., 1993; Ruano et al., 1996), which is consistent with electrostatic incompatibility between protein and acidic lipids at neutral pH. Electrostatic repulsion between DPPG and protein molecules in the monolayer could drive exclusion of SP-A to the observed large protein aggregates into the boundaries between LC and LE domains, regions of the monolayers that are irregularly packed that could act as “sinks” in which lipid-immiscible molecules could most easily accumulate.

Interaction of SP-A with DPPC in the monolayer is consistent with recent results suggesting that SP-A promotes accumulation of DPPC in the surface, perhaps from a sort of DPPC-rich reservoir below the air-water interface (Yu and Possmayer, 1996).

In spite of the immiscibility of SP-A with acidic phospholipids, TR-SP-A still goes into the interfacial monolayers. This could be a consequence of intrinsic surface active properties of the protein itself. Several proteins, particularly glycoproteins, have been reported to possess such intrinsic tensoactive characteristics and can form protein interfacial monolayers by adsorption from the subphase (Ahlers et al., 1990; Heckl et al., 1985; 1987; Mohwald, 1990). We have observed that conA, an acidic glycoprotein that is not
known to interact with phospholipids, also adsorbs into DPPC monolayers with similar aggregation and distribution behavior to TR-SP-A in DPPC/DPPG monolayers. This similarity suggests that the aggregation and accumulation in the LE-LC boundary dislocation is a nonspecific property and that only the different distributions of SP-A in DPPC is specific to these compounds.

Little TR-SP-A fluorescence was observed in interiors of the dark condensed domains of the DPPC monolayers, and some fluorescence was seen in the liquid-expanded regions. King et al. (1983; 1986) have observed that the binding of SP-A to liposomes was greater when the constituent lipids were in the gel state than when they were in the liquid crystalline state. It is noteworthy that there was no appearance of the SP-A molecules at the DPPC monolayer aqueous interface when the lipid is strictly in the liquid expanded phase, which is up to pressures of 3–4 mN/m. Only when condensed domains began to appear did the TR-SP-A appear in the interface. This is consistent with its preferential interaction with gel phase in bilayers or the need for the condensed phase in monolayers. Whereas condensed phase in monolayers, gel phases in bilayers, and lipid-expanded and liquid-crystalline phases are often considered to be roughly equivalent, they are not completely so. The pressures for equivalence, for example, remain under discussion. It seems that the SP-A may be attracted to the dislocations in the condensed-expanded domain and in gel-liquid crystal boundaries, as would any protein with nonspecific binding (e.g., Netz et al., 1996). King et al. (1983) found that binding appeared highest in gel state systems in which dislocations in packing may have been anticipated. The binding of SP-A to DPPC is specific in that it has a different appearance entirely than the binding of SP-A to DPPC/DPPG or of conA to DPPC.

In conclusion, these studies demonstrate that SP-A interacts and perturbs DPPC monolayers, partitioning into the liquid-expanded fluid phase of the phospholipid, and accumulating at the LE-LC boundaries. SP-A causes no effects in monolayers of DPPC/DPPG, the protein being excluded from both LC and LE regions and being accumulated as large protein aggregates in the LE-LC boundaries of these monolayers.

This work has been supported by Medical Research Council of Canada (K.M.W.K.) and Fondo de Investigaciones Sanitarias de la Seguridad Social and Universidad Complutense (C.C. and J.P.G.). Collaboration between Canadian and Spanish groups was facilitated by a Collaborative Research grant from NATO.

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