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Fusion of alphaviruses with liposomes is a non-leaky process

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Abstract It has been reported that low-pH-induced fusion of influenza virus with liposomes results in rapid and extensive release of both low- and high-molecular-weight substances from the liposomes [Günther-Ausborn et al., J. Biol. Chem. 270 (1995) 29279–29285; Shangguan et al., Biochemistry 35 (1996) 4956–4965]. Here, we demonstrate retention of encapsulated water-soluble compounds during fusion of Semliki Forest virus (SFV) or Sindbis virus with liposomes at low pH. Under conditions allowing complete fusion of the liposomes, a limited fluorescence dequenching of liposome-encapsulated calcein was observed, particularly for SFV. Also, radioactively labeled inulin or sucrose were largely retained. Freezing and thawing of the viruses in the absence of sucrose resulted in an enhanced leakiness of fusion. These results support the notion that the alphavirus fusion event per se is non-leaky and may well involve a discrete hemifusion intermediate. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Alphavirus; Hemifusion; Membrane fusion; Semliki Forest virus; Sindbis virus; Virus fusion

1. Introduction

Alphaviruses, such as Semliki Forest virus (SFV) and Sindbis virus (SIN), belong to the category of enveloped viruses that infect their host cells through receptor-mediated endocytosis and subsequent low-pH-induced fusion from within acidic endosomes [1–5]. This fusion process is mediated by the E1 component of the viral E2/E1 heterodimeric envelope glycoprotein [6–12]. SFV and SIN fuse efficiently with liposomes in a strictly low-pH-dependent manner [11,13–15]. Fusion requires the presence of cholesterol [10,11,14,16–18] and sphingolipids in the target membrane [13,14,19,20]. Cholesterol is involved in low-pH-dependent irreversible binding of the virus to target liposomes, while sphingolipids appear to function as a specific cofactor, catalyzing the actual viral membrane fusion process [13].

Fusion of SFV with liposomes was first studied by White and Helenius [16], who used fusion assays based on encapsulation of trypsin or RNase in the liposomes. Efficient degradation of the viral capsid protein or RNA was demonstrated after incubation of SFV with the liposomes at low pH. No RNA degradation was observed when RNase was added to the external medium. Likewise, the presence of an excess of trypsin inhibitor in the external medium does not interfere with degradation of the capsid protein during fusion of SFV or SIN with trypsin-containing liposomes [13,14,16,20]. Although the trypsin and RNase assays do not unequivocally prove the non-leakiness of the fusion process, the results of these studies are consistent with the idea that SFV–liposome fusion is not very leaky to large molecules, such as RNase or trypsin inhibitor. Similar observations have been made for influenza virus. For example, White et al. [21], using the above trypsin assay, and Young et al. [22], studying virus interaction with planar bilayers, reported that the fusion process of influenza virus is non-leaky. However, more recent investigations of influenza–liposome fusion [23–25] strongly suggest that the fusion process is quite leaky. Unlike the study of White et al. [21], these latter investigations are based on the release of reporter molecules from the liposomes during the fusion process. For example, Shangguan and coworkers [25] demonstrated that not only calcein (MW 623) but also 10-kDa dextran is rapidly released from liposomes upon fusion with influenza virus. These observations prompted us to reinvestigate the leakiness of alphavirus–liposome fusion, using similar reporter molecules encapsulated in the liposomes.

2. Materials and methods

2.1. Chemicals

Phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) prepared from egg-PC, sphingomyelin (SPM) from bovine brain, and cholesterol (Chol) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Calcein and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrPC) were from Molecular Probes (Leiden, The Netherlands). [^{3}H]Sucrose and [^{3}H]inulin were from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.2. Viruses

SFV and SIN were produced from baby hamster kidney cells (BHK-21), as described before [11,14]. Virus was purified from cell-culture medium by ultracentrifugation and sucrose-density gradient

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centrifugation in 5.0 mM HEPES, pH 7.4, containing 0.15 M NaCl and 0.1 mM EDTA (HNE). Unless stated otherwise, fresh virus preparations, which had not been frozen and thawed but just stored in the cold, were used. Storage never exceeded 3 days. Viral phospholipid was determined by phosphate analysis [26] after extraction of the lipids according to Bligh and Dyer [27].

2.3. Liposomes

Liposomes (large unilamellar vesicles) were prepared by freeze–thaw extrusion in HNE, as described before [14,28]. The extrusion step was done in a LiposoFast mini-extruder (Avestin, Ottawa, ON, Canada), using first two stacked Nuclepore polycarbonate filters with a pore size of 200 nm and then two stacked filters with a pore size of 50 nm (filters were from Costar, Cambridge, MA, USA). The latter extrusion was done 81 times. The mean diameter of vesicles prepared in this fashion was determined by quasi-elastic light scattering in a Model 370 Submicron Particle Sizer (Nicomp, Santa Barbara, CA, USA), and found to be 70 nm with a narrow size distribution.

Liposomes consisted of either PC/PE/SPM/Chol/pyrPC (molar ratio, 0.85:1.0:1.0:1.5:0.15), PC/PE/SPM/Chol (1.0:1.0:1.0:1.5), PC/PE/Chol/pyrPC (0.85:1.0:1.0:0.15) or PC/PE/Chol (1.0:1.0:1.0). For encapsulation of calcein, liposomes were prepared in 60 mM calcein (sodium salt), 5.0 mM HEPES, 0.1 mM EDTA, pH 7.4. Non-encapsulated calcein was removed by gel filtration on Sephadex G-50 (Pharmacia, Uppsala, Sweden) in HNE. For encapsulation of [^{3}H]sucrose or [^{3}H]inulin, liposomes were prepared in HNE containing 225 μCi/ml [^{3}H]sucrose (13 Ci/mmol) and 1 mM unlabeled sucrose, or 200 μCi/ml [^{3}H]inulin (0.39 Ci/mmol), respectively. Non-encapsulated sucrose or inulin were removed by gel filtration. Phospholipid contents of the liposome preparations were determined by phosphate analysis [26].

2.4. Fusion assay

Lipid mixing during virus–liposome fusion was monitored as a decrease of pyrene excimer fluorescence following acidification of mixtures of SFV or SIN with pyrPC-containing liposomes, as described before [14]. Virus (10 μM phospholipid) and liposomes (2 μM phospholipid) were mixed in 0.66 ml HNE in a quartz microcuvette. Subsequently, the pH of medium was adjusted to 5.5 (for SFV) or 5.0 (for SIN) by addition of 0.040 ml 0.30 M MES, pretitrated to achieve the desired final pH. The time course of pyrene excimer fluorescence was measured at excitation and emission wavelengths of 345 nm and 480 nm, respectively, in the presence of a 475-nm cutoff filter in the emission beam, in an AB2 fluorometer (SLM-Aminco, Urbana, IL, USA). The cuvette holder of the fluorometer was equipped with a magnetic stirring device and maintained at 37°C. The fusion scale was set such that the initial excimer fluorescence intensity represented 0% fusion, and the fluorescence intensity at 33% dilution of the probe represented the 100% value. Complete dilution of the probe was induced by addition of 0.035 ml of 0.20 M octaethyleneglycol monododecyl ether ($C_{12}E_8$; Fluka Chemie AG, Buchs, Switzerland).

2.5. Release of liposome contents during fusion

Release of calcein from the liposomes during fusion with SFV or SIN was determined under conditions as in the fusion assay. Calcein is a water-soluble fluorophore (MW 623) exhibiting fluorescence self-quenching at high concentrations. Thus, leakage of calcein from liposomes containing 60 mM of the probe results in fluorescence dequenching [29]. Calcein fluorescence was measured, in the AB2 fluorometer as above, at excitation and emission wavelengths of 460 nm and 520 nm, respectively, in the presence of a 495-nm cutoff filter in the emission beam. The fluorescence of completely released calcein was determined after the addition of 0.070 ml of 10% (v/v) Triton X-100 (Fluka) to the cuvette.

Leakage of [^{3}H]sucrose was assessed by gel filtration chromatography. Liposomes (10 μM phospholipid) containing [^{3}H]sucrose were mixed with SFV (50 μM phospholipid) in 0.50 ml HNE and acidified as above. After 1 min at pH 5.5 the medium was adjusted to pH 7.8 with 0.018 ml of 0.5 M Tris (pH 9.5). The mixture was loaded on a 24×1 cm Sepharose CL-4B column. The column was eluted with HNE, containing 1 mg/ml bovine serum albumin and 0.1 mM unlabeled liposomes to avoid binding of fusion complexes to the column material. The radioactivity in 0.5-ml fractions was assessed by liquid scintillation counting. Leakage of [^{3}H]inulin during SIN–liposome fusion was determined in a similar manner.

3. Results

3.1. Fusion of SFV or SIN with liposomes assessed by lipid mixing

To determine leakage of liposomal contents during virus–liposome fusion, we used conditions allowing fusion of essentially all of the *liposomes* in a virus–liposome mixture. This condition is achieved with an excess of virus over liposomes, as shown previously for fusion of SIN with liposomes [14]. In this latter study, using 70-nm liposomes, we demonstrated that, at liposome and SIN concentrations of 2 and 2.5 μM phospholipid (corresponding to 4×10^{10} liposomes and 10^{11} virions per ml), a liposome fuses on average once with a single virus particle. Given the diameter of the liposomes of 70 nm and that of an alphavirus particle, excluding the external glycoprotein layer, of 50 nm [12], fusion of a liposome with a virion results in a reduction of pyrPC surface density by 33% and thus in a decrease of pyrene excimer fluorescence by 33%, the excimer intensity being proportional to the surface density of the probe [30]. Likewise, 100% fusion of the liposomes corresponds to a 33% decrease of the total pyrene excimer fluorescence. This value has indeed been observed previously [14].

Now, using pyrPC-labeled PC/PE/SPM/Chol liposomes, again with a diameter of 70 nm, and unlabeled virus at concentrations of 2 and 10 μM, respectively (corresponding to a liposome:virus particle ratio of 1:10), we observed a decrease of pyrene excimer fluorescence intensity in 1 min of 42% for SFV at pH 5.5 (Fig. 1A, curve a) and 37% at pH 5.0 for SIN (Fig. 1B, curve a). These values correspond to 127% and 112% fusion, respectively. Therefore, under the condition of these experiments, on average all of the liposomes fuse at least once with a virus particle. The initial rate of SIN–liposome fusion was approx. two-fold lower than that of SFV–liposome fusion under the condition of the experiment (Fig. 1A vs. 1B).

3.2. Release of calcein from liposomes during fusion with SFV or SIN

Fig. 1A (curve b) shows the result of a calcein dequenching experiment. PC/PE/SPM/Chol liposomes, loaded with 60 mM calcein, were incubated with SFV at pH 5.5, 37°C, at a liposome-to-virus particle ratio of 1:10. Clearly, an only limited dequenching of calcein fluorescence occurred, reaching about 15% after 1 min. Virtually no dequenching was observed at pH 7.4 (not shown), or at pH 5.5 with liposomes lacking SPM (Fig. 1A, curve c). We have shown that fusion of SFV requires the presence of SPM in target liposomes [13]. Therefore, the limited fluorescence dequenching observed with SPM-containing liposomes at pH 5.5 was due to the fusion process.

This dequenching, however, may not be due to release but can possibly be accounted for by redistribution of calcein *within* the liposome–virus fusion products. When a 70-nm liposome fuses with a 50-nm virion and the fusion product assumes a spherical shape, the internal volume of the fusion product increases almost two-fold relative to the internal volume of the original liposome. Thus, we compared the fluorescence of 30 mM calcein to that of 60 mM calcein, both encapsulated in liposomes. The residual relative fluorescence intensities were 30% and 17%, respectively. This implies that a two-fold dilution of the calcein within liposome–virus fusion products would result in an increase of fluorescence intensity

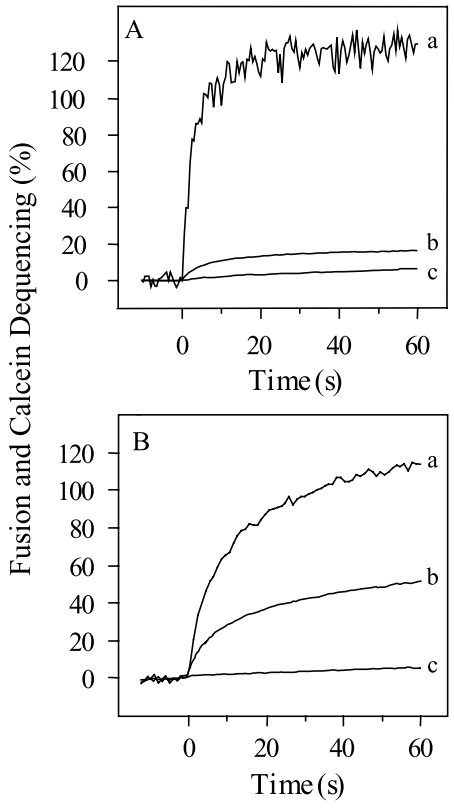

Fig. 1. Fusion of SFV (A) or SIN (B) with pyrPC-labeled or calcein-containing liposomes. Fusion was measured on the basis of pyrene excimer fluorescence decrease, and calcein release on the basis of fluorescence dequenching, both at virus and liposome concentrations of 10 μM and 2.0 μM phospholipid, respectively. The fusion scale is calibrated such that 33% decrease of pyrPC excimer fluorescence intensity represents 100% fusion (see text). The calcein dequenching scale is calibrated such that the initial residual fluorescence of the liposomes represents 0% and the fluorescence intensity in the presence of Triton X-100, inducing lysis of the liposome, 100%. Curves a, fusion of PC/PE/SPM/Chol/pyrPC liposomes with SFV at pH 5.5 or with SIN at pH 5.0; curves b, calcein release from PC/PE/SPM/Chol liposomes upon fusion with SFV at pH 5.5 or SIN at pH 5.0; curves c, calcein release from PC/PE/Chol liposomes at pH 5.5 in the presence of SFV (panel A) or from PC/PE/SPM/Chol liposomes at pH 5.0 in the absence of SIN (panel B).

of $100\times(30-17)/(100-17)=16\%$. The observed dequenching of 15% is similar to this value. Therefore, even though virus–liposome fusion products may not be perfectly spherical, the above argument shows that the observed calcein dequenching is likely to be due, at least in part, to dilution of the probe *within* virus–liposome fusion products.

Fusion of SIN with calcein-loaded liposomes (Fig. 1B) was more leaky than SFV–liposome fusion. With SIN we observed approx. 50% dequenching of calcein under conditions where all of the liposomes fuse at least once with a virion (curve b). This implies that, while SFV–liposome fusion appears to be essentially non-leaky, SIN–liposome fusion results in partial release of calcein to the external medium.

3.3. Retention of radiolabeled sucrose or inulin within virus–liposome fusion products

Final proof for SFV–liposome fusion being essentially non-leaky came from the evaluation of [^{3}H]sucrose release from the liposomes. Liposomes containing [^{3}H]sucrose (MW 344) were incubated with SFV for 1 min at pH 5.5, 37°C, at liposome and virus concentrations of 10 μM and 50 μM phospholipid (liposome-to-virus particle ratio of 1:10). Fig. 2A shows the elution profile of [^{3}H]sucrose obtained after gel filtration analysis of the liposome–SFV fusion. It is evident that only a small fraction (approx. 9%) of the [^{3}H]sucrose had leaked out (closed circles). The controls show that unfused liposomes also retained the [^{3}H]sucrose (open circles), while Triton X-100-treated liposomes completely released the probe (triangles).

Similar experiments were done with SIN and liposomes containing [^{3}H]inulin. Since from the calcein dequenching experiments (Fig. 1B) it was evident that SIN–liposome fusion results in partial release of the probe, we decided to use, rather than sucrose (MW 344) which is smaller than calcein (MW 632), the larger marker inulin (MW 5200). Fig. 2B demonstrates that inulin was largely retained during SIN–liposome fusion, only 11% being released (closed circles).

3.4. Effects of freeze–thawing of the virus on calcein retention

Previous studies [21,22] have indicated that fusion of SFV or influenza virus with planar bilayers or liposomes becomes increasingly leaky when the virus is damaged by freeze–thaw-

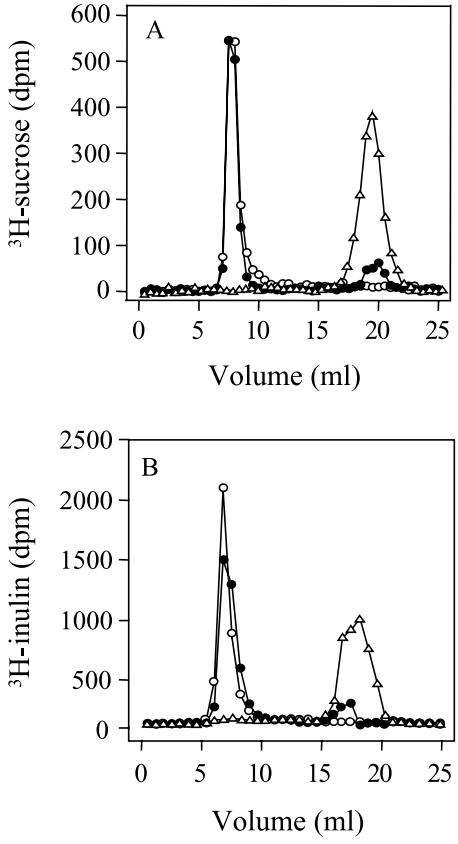

Fig. 2. Release of [^{3}H]sucrose or [^{3}H]inulin from liposomes upon fusion with SFV (A) or SIN (B), respectively. Mixtures of SFV or SIN (50 μM) and PC/PE/SPM/Chol liposomes (10 μM) containing [^{3}H]sucrose or [^{3}H]inulin, respectively, were incubated at pH 5.5 (SFV) or pH 5.0 (SIN), 37°C, for 1 min and subsequently neutralized. The incubation mixtures were then eluted on a Sepharose CL-4B column, and the fractions were analyzed for radioactivity. Open circles, untreated virus–liposome mixtures at neutral pH; closed circles, liposomes after low-pH-induced fusion with SFV or SIN; triangles, virus–liposome mixtures after addition of Triton X-100.

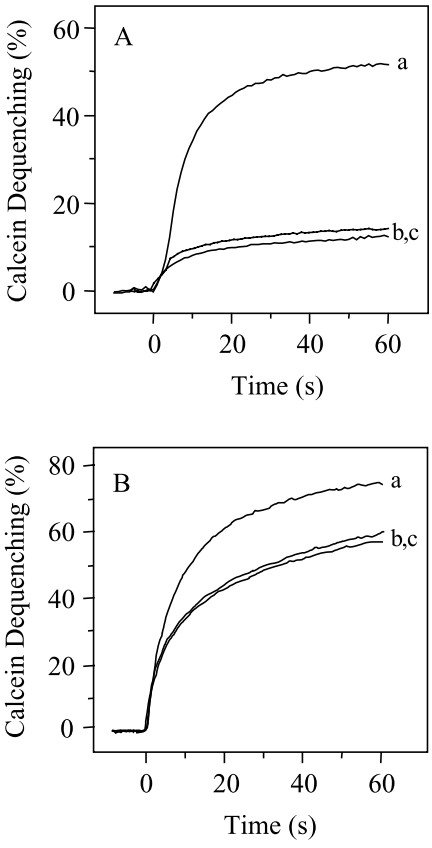

Fig. 3. Effect of prior viral freezing and thawing on calcein release from liposomes upon fusion with SFV (A) or SIN (B). Calcein dequenching was measured as in the experiment of Fig. 1, with virus preparations subjected to freezing (in liquid nitrogen) and thawing (in a waterbath of 37°C). Curves a, virus frozen and thawed once in HNE; curves b, virus frozen and thawed five times in HNE containing 35–40% (w/v) sucrose; curves c, unfrozen virus.

ing. In the above experiments, we used fresh virus preparations which had been stored in the cold for no longer than 3 days. In order to assess the effect of freeze–thawing, aliquots of SFV or SIN, in HNE containing 35–40% sucrose, were subjected to freeze–thawing five times, and subsequently fused to calcein- or pyrPC-containing liposomes. The treatment had no detectable effect on the extents of calcein dequenching (Fig. 3A,B, curves b) or fusion in the pyrene assay (results not shown).

However, when SFV or SIN were frozen and thawed in HNE in the absence of sucrose as a cryopreservative, the fusion process with liposomes did become leaky, resulting in 55% calcein dequenching with SFV (Fig. 3A, curve a). For SIN, similar results were obtained (Fig. 3B, curve a), but the effect of freeze–thaw treatment was much less prominent than with SFV. This indicates that freezing and thawing in the absence of a cryopreservative may induce structural defects in the virus membrane, resulting in leakage of liposome contents after fusion of such a damaged virus with liposomes.

4. Discussion

The results of this study indicate that fusion of alphaviruses with liposomes, particularly fusion of SFV, is a relatively non-leaky process. Early studies on SFV–liposome fusion, employing an assay based on trypsin or RNase encapsulated in the liposomes, had provided circumstantial evidence for the fusion event to be non-leaky to large molecules [16]. Our present observations, based on direct evaluation of the release of marker molecules from the liposomes, extend this notion by demonstrating that even small molecules, such as calcein and sucrose, are largely retained during the fusion process. The initial integrity of the viral membrane appears to be crucial, as prior freeze–thaw treatment of the viruses in the absence of sucrose resulted in an increased leakiness. This suggests that, when release of marker molecules from target liposomes is observed during alphavirus–liposome fusion, it may not be due to the fusion process per se, but rather the result of the presence of structural defects in the viral membrane. It is interesting that Young et al. [22], studying fusion of SFV with planar lipid bilayers, also concluded that fusion of SFV is a non-leaky process provided that the virus is fresh and the viral envelope undamaged. Furthermore, SFV or SIN have been observed to become hemolytic only when the viruses are subjected to freeze–thaw treatment prior to incubation with the erythrocytes [31].

There appeared to be a difference between the degrees of leakiness of SFV- and SIN-liposome fusion, the latter resulting in a more extensive release of calcein (Fig. 1). At the same time, freeze–thaw treatment of SIN had a comparatively small effect (Fig. 3B). This suggests that the SIN virus preparation, although freshly and carefully isolated, may have acquired more structural defects during the purification process than SFV. Yet, SIN–liposome fusion was essentially non-leaky to the marker molecule inulin (MW 5200).

Our results are in apparent disagreement with observations by Spyr et al. [32] and Käsermann et al. [33], indicating that, at low pH, isolated SFV particles become permeable to small molecules, such as propidium iodide. The authors propose that the viral E1 protein forms non-specific pores in the viral membrane under acidic conditions. Accordingly, one would expect that, upon virus–liposome fusion, small molecules initially encapsulated in the liposomes would be released to the external medium through these pores. However, since we did not observe such release, it would appear that the pores are either not or only transiently formed under the conditions of our experiments or that they are not accessible, possibly due to blocking by the viral nucleocapsid.

It is remarkable that, while alphavirus–liposome fusion is essentially tight, influenza–liposome fusion has been found to be leaky not only to calcein but also to 10-kDa dextran [23–25]. It is unclear what the basis for this difference is. However, the following points can be made. There is convincing evidence to indicate that fusion mediated by the influenza hemagglutinin involves a hemifusion intermediate, a so-called stalk [34–37]. Conceptually, a distinct hemifusion intermediate, in which the outer leaflets of the interacting membranes have merged while the inner leaflets are still separate, would provide a very plausible explanation for a fusion process being non-leaky. Therefore, the observed leakiness of influenza–liposome fusion [24,25] may not be due to leakiness of the fusion process per se, but rather to preexisting structural defects in the viral membrane, as discussed above, or to a secondary effect of virus–liposome fusion. It is interesting that influenza virus fusion is strongly hemolytic [38] whereas the fusion event with erythrocytes per se, involving a hemifusion intermediate, may well be non-leaky.

At the same time, the relative non-leakiness of alphavirus–liposome fusion supports the notion that this fusion involves a distinct hemifusion intermediate. In this regard, we have characterized the effects of lysophosphatidylcholine and free fatty acids on SFV–liposome fusion and observed that these effects indeed strongly suggest that the fusion reaction proceeds via a stalk mechanism (Ortiz et al., to be submitted).

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