# Membrane Phosphatidylserine Regulates Surface Charge and Protein Localization

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# Membrane Phosphatidylserine Regulates Surface Charge and Protein Localization

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Electrostatic interactions with negatively charged membranes contribute to the subcellular targeting of proteins with polybasic clusters or cationic domains. Although the anionic phospholipid phosphatidylserine is comparatively abundant, its contribution to the surface charge of individual cellular membranes is unknown, partly because of the lack of reagents to analyze its distribution in intact cells. We developed a biosensor to study the subcellular distribution of phosphatidylserine and found that it binds the cytosolic leaflets of the plasma membrane, as well as endosomes and lysosomes. The negative charge associated with the presence of phosphatidylserine directed proteins with moderately positive charge to the endocytic pathway. More strongly cationic proteins, normally associated with the plasma membrane, relocalized to endocytic compartments when the plasma membrane surface charge decreased on calcium influx.

The negative surface charge of the inner leaflet of the plasma membrane determines the targeting of proteins containing polycationic motifs (*1*). The unique negativity of the plasmalemmal inner leaflet has been attributed, in part, to its high polyphosphoinositide content. Phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] and phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$] are required to target and retain polycationic proteins, such as K-Ras, to the plasma membrane (*2*). Although they are polyvalent, polyphosphoinositides represent only a minor fraction of the phospholipids of the plasma membrane and are less abundant than phosphatidylserine (PS), the predominant anionic species, which represents 10 to 20% of all surface lipid (*3*). The extent to which PS contributes to the targeting and retention of cationic proteins in cells is unclear, because suitable probes to monitor PS distribution in live cells are lacking.

We wanted to develop a probe to monitor the endogenous distribution of PS in intact cells. Lactadherin, a glycoprotein of milk, binds PS in a calcium-independent manner (*4*, *5*), with the major PS-binding motif localized to its C2 domain. We used the C2 domain of lactadherin (Lact-C2) to generate genetically encoded fluorescent biosensors of PS (*6*). Recombinant Lact-C2 was generated in bacteria and purified to test its affinity for PS-containing liposomes by fluorescence resonance energy transfer (*7*) (fig. S1, A and B). We assessed the effectiveness of liposomes of varying composition to displace Lact-C2 from glass microspheres coated by phospholipid bilayers containing 20% PS and 80% phosphatidylcholine (PC) (lipospheres). Liposomes containing 5 to 20% PS reduced Lact-C2 binding to the lipospheres in a concentration-dependent manner (fig. S1C), whereas liposomes containing only PC (fig. S1C) or physiological levels of either phosphatidylinositol (PI) or $PI(4,5)P_2$ had no effect (Fig. 1A). Lact-C2 fused to green fluorescent protein (GFP–Lact-C2) also bound exclusively to PS-coated beads but not to those coated with PC or phosphatidylethanolamine (PE), or with anionic phospholipids such as phosphatidic acid (PA), PI, or $PI(4,5)P_2$ (Fig. 1B), which confirmed the selectivity observed with the bacterially expressed C2 domain.

We next expressed GFP–Lact-C2 in both wild-type and mutant *Saccharomyces cerevisiae* deficient in PS (*8*). GFP–Lact-C2 was observed predominantly on the plasma membrane of the wild-type yeast (Fig. 1C); however, it was cytosolic in the PS-deficient mutant (Fig. 1C). Although a contribution of protein-protein interactions cannot be completely ruled out, these findings demonstrated that most membrane binding of Lact-C2 requires PS.

The plasma membrane of RAW264.7 macrophages was similarly labeled by the GFP–Lact-C2 probe (Fig. 1D). Additionally, intracellular vesicles were decorated with GFP–Lact-C2 (Fig. 1D), and a similar distribution was noted for the C2 domain of factor VIII, which also binds PS (*9*) (fig. S3A). Certain key residues in the C2 domains of factors VIII and V (fig. S2) are required for these proteins to bind PS. Mutation of the equivalent residues in GFP–Lact-C2 obliterated its ability to bind PS in vitro (Fig. 1B) and rendered the construct cytosolic in macrophages (Fig. 1D).

Several lines of evidence indicated that membrane binding of GFP–Lact-C2 was driven specifically by PS and not by negative charge. First, plasmalemmal targeting of GFP–Lact-C2 persisted after $PI(4,5)P_2$ was depleted by either synaptojanin 2 (fig. S4A) or Inp54 (an inositol 5-phosphatase) (fig. S4, B and C). Lact-C2 remained bound to the membrane, despite elimination of $PI(3,4,5)P_3$ with wortmannin (fig. S4, A and C) or when

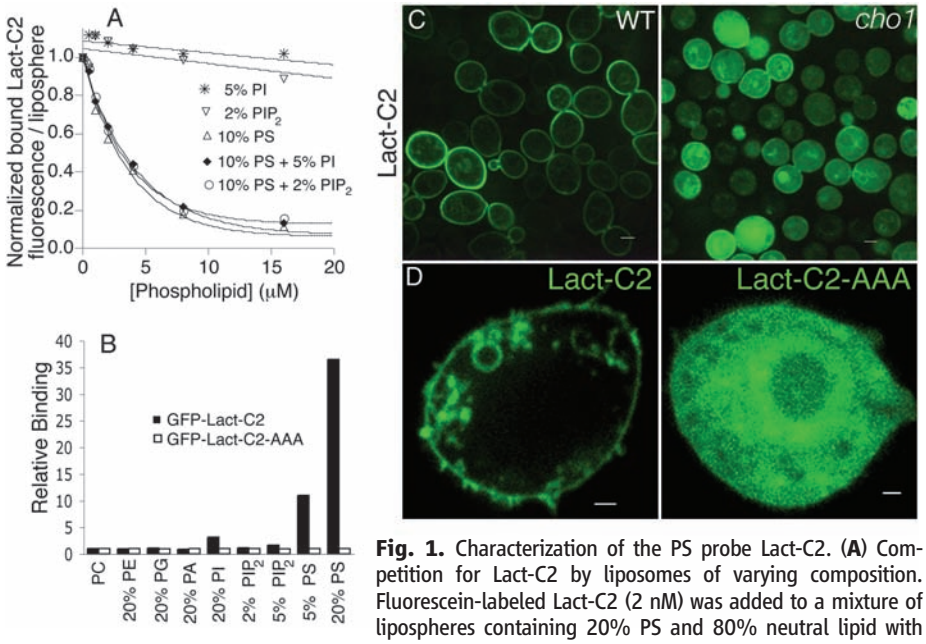


**Fig. 1.** Characterization of the PS probe Lact-C2. (**A**) Competition for Lact-C2 by liposomes of varying composition. Fluorescein-labeled Lact-C2 (2 nM) was added to a mixture of lipospheres containing 20% PS and 80% neutral lipid with increasing concentrations of liposomes containing the indicated mole fraction of anionic lipids (the balance was PC). (**B**) Binding of wild-type GFP–Lact-C2 (black bars) or GFP–Lact-C2-AAA (*6*, *15*) (white bars) partially purified from HeLa cells to C18-nucleosil beads coated with PC alone or with PE (20%), PG (20%), PA (20%), PI (20%), $PI(4,5)P_2$ ($PIP_2$) (2%, 5%), or PS (5%, 20%). Data expressed relative to PC-only beads. (**C**) Confocal images of wild-type *S. cerevisiae* (left) and a PS-deficient mutant (*cho1*) (right) expressing GFP–Lact-C2. Scale bars here and elsewhere are 2 μm. (**D**) Confocal images of RAW264.7 macrophages expressing GFP–Lact-C2 (left) or GFP–Lact-C2-AAA (right).

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$PI(4,5)P_2$ and $PI(3,4,5)P_3$ were eliminated by adenosine 5′-triphosphate (ATP) depletion (fig. S4, D and E). Similarly, the accumulation of Lact-C2 on endomembranes remained unchanged when phosphatidylinositol 3-phosphate [PI(3)P] was depleted (fig. S4F). Last, reducing the negativity of the plasma membrane by addition of sphingosine (a membrane-permeant base) or squalamine (a divalent cationic sterol) was without effect on GFP–Lact-C2, but induced a drastic redistribution of surface charge reporters (fig. S5, A and B) (*10*).

Next, we identified the endomembrane organelles bearing PS on their cytosolic surface by colocalization analysis. Mitochondria (Fig. 2A), the Golgi complex (Fig. 2B), and the endoplasmic reticulum (Fig. 2C) did not colocalize significantly with Lact-C2. These findings were unexpected because PS is synthesized, transported, or metabolized in these compartments. The concentration of PS in organelles of the secretory pathway may be lower than that of the plasma membrane, and/or PS may be largely confined to their luminal leaflet. In contrast, extensive overlap was observed between the endocytic pathway and Lact-C2 (Fig. 2D). Various subcompartments of the endocytic pathway were labeled with Lact-C2; early endosomes (fig. S6, A and B), late endosomes (fig. S6C), and lysosomes (fig. S6D) all colocalized with the PS probe.

The presence of PS, an anionic lipid, may confer negative surface charge to endosomes and lysosomes. To test this hypothesis, we expressed a series of surface charge biosensors. These probes combine a hydrophobic farnesyl chain with an adjacent sequence of varying net positive charge (*11*). The most cationic of these biosensors (8+), which is expected to accumulate on membranes with the most-negative surface charge, localized preferentially to the plasmalemma (Fig. 3A), which supports the notion that this membrane bears the most negativity on its inner surface, likely because of the unique accumulation of phosphoinositides (*1*, *12*, *13*). Accordingly, the 8+ probe was observed to relocalize from the plasma membrane to internal organelles after $PI(4,5)P_2$ and $PI(3,4,5)P_3$ depletion (fig. S5C).

By contrast, the least cationic probe (2+), which is targeted predominantly by hydrophobic interactions, associated mainly with intracellular membranes (Fig. 3A). A progressive increase in the number of positive charges should favor interaction of the biosensors with more negatively charged membranes. Indeed, the fraction of the fluorescence associated with the plasma membrane increased gradually along with the charge of the probes (Fig. 3, A and B). This was accompanied by redistribution of the fluorescence in endomembranes. Whereas the farnesylated 2+ probe clearly associated with reticular and juxtanuclear structures, which feature prominently components of the secretory pathway, the 6+ probe bound to the plasmalemma and to a more discrete, vesicular subpopulation (Fig. 3A). A 4+ probe showed an intermediate distribution. Thus, the surface charge of the reticular and juxtanuclear membranes may be less negative than that of the more randomly distributed vesicular membranes.

The nature of the vesicular compartment and the source of its negative surface charge were revealed when we compared the distribution of the charge biosensors with that of Lact-C2. The

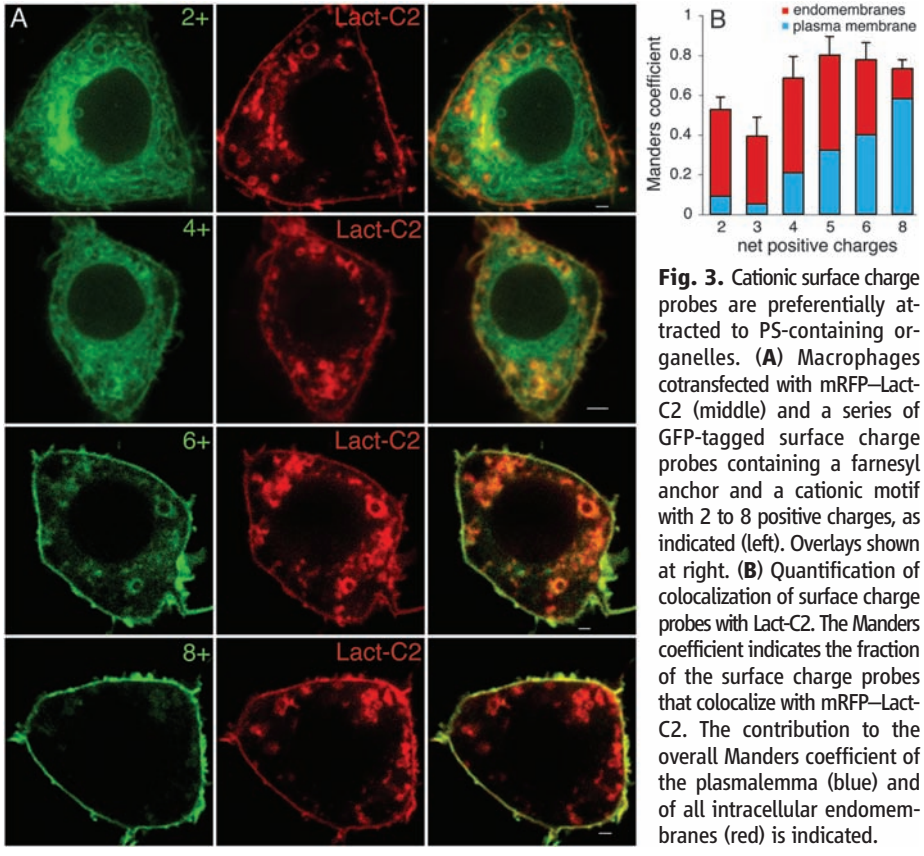



**Fig. 3.** Cationic surface charge probes are preferentially attracted to PS-containing organelles. (**A**) Macrophages cotransfected with mRFP–Lact-C2 (middle) and a series of GFP-tagged surface charge probes containing a farnesyl anchor and a cationic motif with 2 to 8 positive charges, as indicated (left). Overlays shown at right. (**B**) Quantification of colocalization of surface charge probes with Lact-C2. The Manders coefficient indicates the fraction of the surface charge probes that colocalize with mRFP–Lact-C2. The contribution to the overall Manders coefficient of the plasmalemma (blue) and of all intracellular endomembranes (red) is indicated.

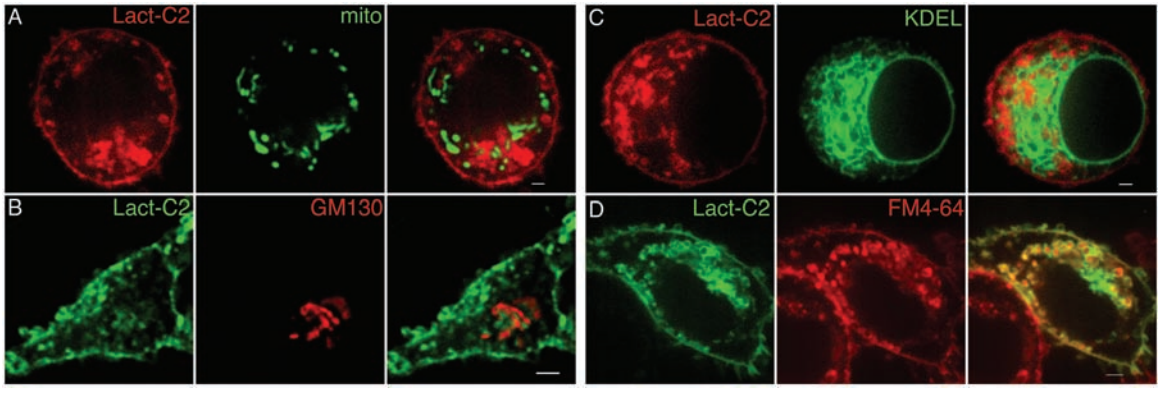



**Fig. 2.** Subcellular distribution of PS in macrophages. (**A**) Macrophage coexpressing the monomeric form of red fluorescent protein (mRFP)–Lact-C2 (left) and mitochondria-targeted GFP (mito-GFP) (middle). Overlays of both images shown in right panel here and below. (**B**) Macrophages expressing GFP–Lact-C2 (left) were stained with antibodies to GM130 (middle). (**C**) Macrophages coexpressing mRFP–Lact-C2 (left) and the endoplasmic reticulum marker KDEL-GFP (middle). (**D**) Macrophages expressing GFP–Lact-C2 (left) incubated 45 min with FM4-64 to label the endocytic pathway (middle).

compartments of intermediate negative surface charge (lower than that of the plasmalemma, but higher than that of secretory membranes) identified by the 6+ probe were clearly labeled by Lact-C2 (Fig. 3A). Surface charge biosensors with progressively lower positive charge showed steadily decreasing colocalization with the PS probe (Fig. 3, A and B). This implies that the cytosolic leaflet of endosomes and/or lysosomes is negative and that the charge is conferred, at least in part, by PS.

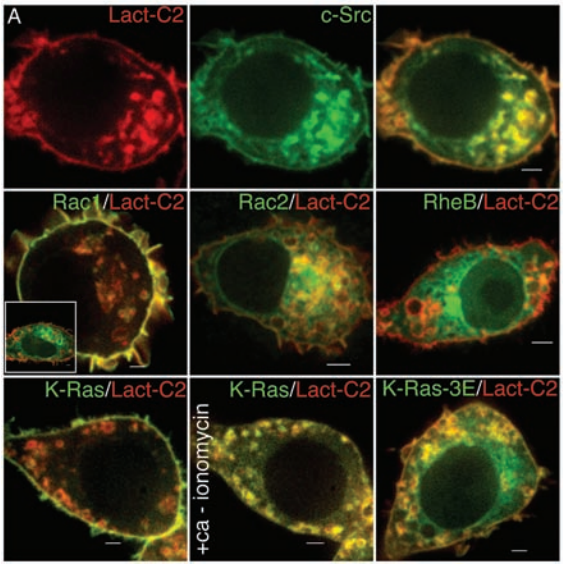
Two additional lines of evidence indicate that PS contributes to the recruitment of charge biosensors to the membranes. First, in ATP and phosphoinositide-depleted cells, the 8+ probe partially relocalized from the plasma membrane to internal organelles (fig. S5C). The redistribution was not random; the 8+ probe was targeted to internal membranes that bound Lact-C2 (fig. S5C). Moreover, a considerable fraction of Lact-C2 remained at the plasmalemma, despite the fact that $PI(4,5)P_2$ and $PI(3,4,5)P_3$ were no longer detectable (fig. S4, D and E). This suggests that PS, which persists in the membrane of ATP-depleted cells, is responsible for retention of the +8 probe. Accordingly, we found that, although the +8 probe is partially retained on the surface membrane of wild-type yeast after ATP depletion, it is largely lost from the membrane of PS-deficient yeast (fig. S7).

The finding that PS-enriched endomembranes recruit proteins bearing sequences with a moderately cationic charge has important functional implications. Although the plasma membrane is the platform where most signal transduction events are initiated, endomembranes are increasingly recognized as sites where signals can be further propagated and amplified (*14*). We therefore analyzed whether the negative charge associated with the enrichment of PS in endocytic compartments contributes to the recruitment of signaling molecules to these loci. Particular attention was given to molecules that, like our charge biosensors, have both a hydrophobic membrane-binding moiety and a cationic motif of intermediate (3+ to 6+) charge. Several well-known signaling molecules fit this description. c-Src, which contains 5+ charges at its myristoylated N terminus, was found at the plasma membrane and in a vesicular compartment that overlapped extensively with Lact-C2 (Fig. 4 and fig. S8). The active forms of Rac1 and Rac2 were similarly found in PS-containing membranes. Of note, the more cationic Rac1 (6+) associated predominantly with the plasmalemma, with a smaller fraction in endomembranes, whereas the converse was true for the less charged (3+) Rac 2 (Fig. 4, A and B). That Rac1 is directed to the surface membrane by its cationic tail is suggested by the observation that a mutant where all six charges were neutralized by replacement of tail cationic amino acids with glutamine (Q) (Rac1-6Q) (*15*) had a widespread distribution reminiscent of the 2+ probe (Fig. 4A). The colocalization of moderately cationic proteins with PS-enriched membranes extends to other Rho family proteins like Cdc42 and to members of the Ras and Rab families (fig. S9). By contrast, guanosine triphosphatases like RheB that bear no positive charge in their tail partition indiscriminately to most cell membranes, setting an upper limit for purely coincidental colocalization with Lact-C2 (Fig. 4).

The electrostatic interaction that targets signaling molecules to PS-enriched endomembranes can be modulated by varying the surface charge of the membrane or by altering the net charge on the protein. K-Ras, which carries 8+ charges at its C-terminal tail, is located primarily at the plasma membrane in resting cells (*16*) (Fig. 4A). When cytosolic calcium rises, plasmalemmal PS is externalized (*17*) and $PI(4,5)P_2$ is hydrolyzed. As a result, the plasmalemmal surface charge decreases and becomes comparable to that of endomembranes, which now compete effectively for binding of K-Ras (Fig. 4, A and B). Conversely, post-translational modifications, like phosphorylation, that reduce the net charge of the cationic tail of K-Ras can also alter its localization (*18*). Accordingly, a phosphomimetic K-Ras mutant with three serine- or threonine-to-glutamate substitutions in its polybasic domain (K-Ras-3E) relocated from the plasmalemma to endomembranes, notably those enriched in PS (Fig. 4).

The presence of sizable pools of PS on the cytosolic leaflet of endosomes and lysosomes implies that these compartments can serve to dock proteins with PS-binding C2-domains, which include a number of important signaling and fusogenic effectors. The accumulation of the anionic lipid also produces the accretion of negative surface charge. As a result, polycationic proteins, particularly those bearing a hydrophobic anchorage site, associate with PS-enriched compartments, including endosomes and/or lysosomes. Electrostatic binding will occur in a manner dependent on both the charge of the membrane and that of the ligand, such that the most-negative membrane (i.e., the plasmalemma) will overwhelmingly accumulate the most cationic proteins, whereas less-positive proteins associate with the plasma membrane and, to a substantial degree, also with membranes of intermediate charge. Because the interaction is dynamic, changes in charge can redirect proteins from one target membrane to another. Thus, diminution of the plasmalemmal charge caused by phospholipid redistribution or metabolism, or phosphorylation of proteins like K-Ras can relocalize them to endocytic membranes, where they could catalyze a different set of reactions.

Clearly, interaction with the surface charge of membranes is but one of the determinants of protein targeting, and other types of interactions must not be neglected. However, the contribution of electrostatic attractions, particularly in endomembranes, should be reevaluated. The electrostatic switch theory (*19*) may be extended to include intermembrane redistribution of ligands in response to charge alteration.



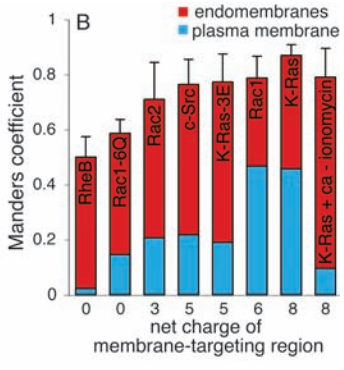




**Fig. 4.** Proteins with cationic domains are recruited to PS-containing compartments. (**A**) Macrophages coexpressing mRFP–Lact-C2 (red) and one of the following: c-Src–GFP, GFP-Rac1(Q61L), GFP–Rac1(Q61L)-6Q (inset), cyan fluorescent protein (CFP)-Rac2(Q61L), CFP-RheB, GFP–K-Ras(V12)-3E (activated Ras with two tail Ser and one Thr replaced by Glu to mimic phosphorylation), or GFP–K-Ras (green) (*6*). (Top, right) Overlay of mRFP–Lact-C2 and c-Src–GFP. Where indicated, cells were treated with ionomycin. (**B**) Quantification of protein colocalization with the PS probe, measured as the Manders coefficient. The contribution of the plasma membrane (blue) versus that of endomembranes (red) is indicated.

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**Supporting Online Material**
www.sciencemag.org/cgi/content/full/319/5860/210/DC1
Materials and Methods
Figs. S1 to S9
References

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# The Limits of Counting: Numerical Cognition Between Evolution and Culture

**Sieghard Beller and Andrea Bender***

Number words that, in principle, allow all kinds of objects to be counted ad infinitum are one basic requirement for complex numerical cognition. Accordingly, short or object-specific counting sequences in a language are often regarded as earlier steps in the evolution from prematheматical conceptions to greater abstraction. We present some instances from Melanesia and Polynesia, whose short or object-specific sequences originated from the same extensive and abstract sequence. Furthermore, the object-specific sequences can be shown to be cognitively advantageous for calculations without notation because they use larger counting units, thereby abbreviating higher numbers, enhancing the counting process, and extending the limits of counting. These results expand our knowledge both regarding numerical cognition and regarding the evolution of numeration systems.

The discovery of the largely restricted (*1*) or probably even nonexistent (*2*) numeration system of the Pirahã in the Amazonian Basin contributed to the discussion of how numerical cognition depends on language. Numeration systems are cognitive tools for numerical cognition (*3*–*6*), and the experimental evidence gathered among the Pirahã provided a sound basis for an analysis of how such tools interact with the cognitive processing of numbers. Cognitive tools, like tools in general, may be more or less efficient, and respective differences in efficiency have been demonstrated both for notational (*7*, *8*) and for purely linguistic numeration systems (*9*–*12*). It should be noted, however, that the assessment of whether a feature is efficient always depends on the nature of the task and on the context of usage and that the efficiency of a specific numeration system does not say anything about the cognitive abilities of its users.

Apart from their efficiency, cognitive tools can also be ordered according to their presumed evolution. Because tools are typically developed in order to improve their efficiency, it is reasonable to assume that numeration systems evolve from being simpler to more sophisticated (*6*, *13*–*15*). But can one also conclude that the simpler a numeration system, the older it is? Although the authors of the recent studies on the Amazonian cases were careful not to draw this conclusion, the evolutionary status of the Pirahã system has become a matter of lively debate, both inside (*2*) and outside of academia. We propose that drawing conclusions on the cognitive and evolutionary status of specific numeration systems requires both diachronic and synchronic data. We set out to highlight the cognitive efficiency of some allegedly primitive systems in another part of the world and to show how they may have evolved from abstract to more specific as a result of cultural adaptation.

Among the properties commonly taken as indices for the simplicity of a numeration system are its extent and its degree of abstractness. The two are largely independent of each other, both on theoretical grounds as well as in practice, and they differ in terms of the attention they have attracted: Whereas the extent of numeration systems has been extensively addressed recently (*1*, *2*, *12*), the degree of abstractness has largely been neglected so far. We will illustrate these properties with two instances for each but will focus on the second feature.

One region where systems with limited extent abound is Papua New Guinea (*16*). Takia, a language in Madang Province, contains five numerals—*kaik*, *uraru*, *utol*, *iwaiwo*, and *kafe-n* (also denoting "his/her thumb"). Higher numbers may be composed by adding or multiplying numerals to the word for 5, but this seems to have been done rarely and for low numbers only (*17*). Adzera, a related language in the Markham River valley in Morobe Province, contains an even more restricted system. Its number words for 1 to 5 are composed of numerals for 1 and 2 only: *bits*, *iru*$^{?}$, *iru*$^{?}$ *da bits* (= 2 + 1), *iru*$^{?}$ *da iru*$^{?}$ (= 2 + 2), and *iru*$^{?}$ *da iru*$^{?}$ *da bits* (= 2 + 2 + 1). Although because of its recursive character this system is in principle infinite, the inevitable difficulties in tallying the terms in higher-number words render it cumbersome. In such cases, people nowadays prefer to use loan words from Tok Pisin instead, a creole language based on English and used as lingua franca in New Guinea (*18*).

These two numeration systems are admittedly not as simple as the case of the Pirahã system, but their low bases and the lack of higher powers of their base restrict both of them. Although numerical cognition among the two Melanesian groups has not been studied experimentally, it can be inferred by analogy that, with such restricted systems, precise numerical operations should be laborious, if not impossible, for larger numbers (*1*, *12*, *19*).

The second property that is readily taken as evidence for restricted efficiency of a numeration system is its object specificity. Menninger inferred that the more object-specific counting sequences a language contains, the more antiquated the numeration system is (*14*). One of the languages referred to as having such object-specific counting sequences is Old High Fijian, a language in the eastern part of Fiji: Whereas it denotes 100 as *bola* when canoes are counted, for coconuts *koro* is used (*20*). Similar object-specific counting sequences can be found in the related Polynesian languages. On Mangareva, for instance, a volcanic island group in French Polynesia, tools, sugar cane, pandanus, breadfruit, and

**Table 1.** Numerals in traditional Mangarevan (abstract sequence).

| Single numerals | | | | Power numerals (quantities) | | | |
|---|---|---|---|---|---|---|---|
| 1 | tahi | 6 | ono | $10^1$ | rogo'uru | $2 \cdot 10^5$ | makiukiu |
| 2 | rua | 7 | hitu | $2 \cdot 10^1$ | takau | $2 \cdot 10^6$ | makore |
| 3 | toru | 8 | varu | $2 \cdot 10^2$ | rau | $2 \cdot 10^7$ | makorekore |
| 4 | hā | 9 | iva | $2 \cdot 10^3$ | mano | $2 \cdot 10^8$ | tini |
| 5 | rima | | | $2 \cdot 10^4$ | makiu | $2 \cdot 10^9$ | maeaea |

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