Arf, Sec7 and Brefeldin A: a model towards the therapeutic inhibition of guanine nucleotide-exchange factors

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Abstract
GEFs (guanine nucleotide-exchange factors), which stimulate GDP dissociation from small G-proteins, are pivotal regulators of signalling pathways activated by small G-proteins. In the case of Arf proteins, which are major regulators of membrane traffic in the cell and have recently been found to be involved in an increasing number of human diseases, GDP/GTP exchange is stimulated by GEFs that carry a catalytic Sec7 domain. Recent structural results captured snapshots of the exchange reaction, revealing that Sec7 domains secure Arf-GDP to membranes before nucleotide exchange takes place, taking advantage of a built-in structural device in Arf proteins that couples their affinity for membranes to the nature of the bound nucleotide. One of the Arf–Sec7 intermediates was trapped by BFA (Brefeldin A), an uncompetitive inhibitor of Arf activation that has been instrumental in deciphering the molecular principles of membrane traffic at the Golgi. BFA targets a low-affinity Arf–Sec7 intermediate of the exchange reaction. It binds at the Arf-GDP/Sec7 interface, thus freezing the complex in an abortive conformation that cannot proceed to nucleotide dissociation. In the cell, this results in the specific inhibition of Arf1 by a subset of its GEFs, and the efficient and reversible block of membrane traffic at the Golgi. The mechanism of BFA leads to the concept of ‘interfacial inhibition’, in which a protein–protein interaction of therapeutic interest is stabilized, rather than impaired, by a drug. Up-regulated activity of small G-proteins is involved in various human diseases, making their GEFs attractive candidates to interrupt specifically the corresponding signalling pathway. Interfacial inhibitors are proposed as an alternative to competitive inhibitors that may be explored for their inhibition.

Activation of Arf by Sec7 domains in three dimensions
Small G-proteins of the Arf family regulate various aspects of cellular traffic through their GDP/GTP cycle and the ability of their GTP-bound form to interact with multiple effectors [1]. Their best characterized functions are the recruitment of coat polymers involved in vesicular traffic at the Golgi (COP1 coats, clathrin adaptors) and the activation of membrane-modifying enzymes (phospholipase D and phosphatidylinositol kinases) [2], and in the case of Arf6 a possibly distinct role in co-ordinating membrane traffic to cytoskeletal dynamics which is less well characterized at the molecular level [3]. Conversion of Arf proteins from an inactive, GDP-bound form into an active, GTP-bound form is stimulated by GEF (guanine nucleotide-exchange factors; ArfGEFs), a family of proteins characterized by a conserved catalytic domain of approx. 200 amino acids called the Sec7 domain [4]. An essential role of ArfGEFs in defining the spatiotemporal conditions of Arf activation – and possibly its outcome – is suggested by several observations, including the fact that a particular Arf protein activated by different ArfGEFs targets to different subcellular membranes and recruits distinct effectors. This is expected to result from the specificity of the Sec7 domain for particular Arf proteins, and/or from the non-catalytic domains, which classify ArfGEFs into different subfamilies but have, in most cases, unknown functions [5].

The Sec7 domain has fostered most efforts so far, including in-depth analysis at the structural and biochemical level. A recent series of structural snapshots of Arf–Sec7 complexes undergoing nucleotide exchange allowed the reconstitution for the first time a ‘three-dimensional movie’ of a GDP/GTP exchange reaction [6]. Arf proteins feature a built-in structural device that allows them to couple their activation by GDP/GTP exchange to their attachment to membranes [7] and Sec7 domains, as they require membranes to be active, were recognized early to be involved in monitoring this coupling [8]. As with all small G-proteins, activation of Arf by its ArfGEFs initiates with the low-affinity docking of Arf-GDP on to its GEF, triggering a series of conformational changes leading to a high-affinity, nucleotide-free Arf–Sec7 complex. This complex then binds GTP, which is more abundant than GDP in the cell, and eventually dissociates to release active Arf-GTP for its subsequent interaction with effectors. Small G-protein–GDP–GEF complexes have in general too low an affinity to be crystallized, but in the case of Arf two such complexes can be trapped by either BFA (Brefeldin A), an uncompetitive inhibitor of Arf at the Golgi [9,10], or a charge reversal mutation of the invariant catalytic glutamate [11].

Key words: Arf–Sec7, Brefeldin A (BFA), Golgi, guanine nucleotide-exchange factor (GEF), membrane traffic, small G-binding protein
Abbreviations used: BFA, Brefeldin A; GEF, guanine nucleotide-exchange factor
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The GDP/GTP structural cycle of Arf proteins

Crystallographic structures of Arf-GDP [12], BFA-trapped Arf-GDP–Sec7 [6,13], charge-reversal-trapped Arf-GDP–Sec7 [6], nucleotide-free Arf–Sec7 [14] and Arf-GTP [14] now illuminate the dynamics of the exchange reaction at the atomic level. The complete picture emerges from combining these structural results with biochemical studies, elucidating the role of membranes as ‘cofactors’ of Arf activation [8,11]. Altogether, these studies reveal that Sec7 domains have the dual functions of securing Arf-GDP to membranes and stimulating GDP dissociation, and that these events take place in an ordered sequence. Nucleotide dissociation can only take place after Arf-GDP is strongly associated with a membrane, thus ensuring that Arf-GTP does not form in the cytosol. The Sec7 domain is acting essentially as a rigid platform throughout the reaction, allowing Arf to rotate from a docking position where the catalytic machinery of the Sec7 domain is far from GDP, then to an intermediate where the catalytic glutamate is poised to undergo electrostatic repulsion of the nucleotide, and finally to a position in which the Glu finger mimics nucleotide phosphates in the, now empty, site. Thus, the spectacular conformational changes of Arf, which involve several β-strand breaks and/or formation and the remodelling of an entire face of the protein (Figure 1A), can take place without unfolding of the Arf protein which remains ‘chaperoned’ by the GEF until the reaction is complete. At the cellular level, these structures establish a consistent picture for the recruitment of Arf-GTP to subcellular membranes where it encounters its effectors [6].

**Towards GEFs as therapeutic targets for interfacial inhibitors**

The unusual uncompetitive mechanism of BFA, in which a small molecule binds at the interface of a macromolecular complex in the course of structural transitions from a stable conformation to another, was termed ‘interfacial inhibition’ [6]. BFA is not the only one of a kind and small molecules, mostly natural products such as the anticancer agents camptothecin (which inhibits a topoisomerase 1–DNA complex) or colchicin (which targets tubulin), are increasingly being characterized as potential interfacial inhibitors of macromolecular interactions. Nature thus shows us an almost unexplored alternative to competitive inhibition for the discovery of drugs that inhibit protein interactions [19]. From this point of view, interfacial inhibitors have potential advantages over competitive inhibitors: (i) binding sites for small molecules are most likely to occur at macromolecular interfaces, whose topology features concave surfaces, than

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**Figure 1** | The GDP/GTP structural cycle of Arf proteins

(A) Structural plasticity of Arf proteins: structures of Arf in Arf-GDP (yellow), Arf-GDP–BFA–Sec7 (red), Arf-GDP–Sec7 [E2005 Biochemical Society]

(B) Structural plasticity of Arf proteins: structures of Arf in Arf-GDP (yellow), Arf-GDP–BFA–Sec7 (red), Arf-GDP–Sec7 [E2005 Biochemical Society] (pink), nucleotide-free Arf–Sec7 (violet) and Arf-GTP (cyan) are superimposed based on their common core. Note the conformational changes at the switch 1 and 2, which are structural sensors of the GTP γ-phosphate, and the β-strand register shift at the interswitch that occurs between steps 2 (red) and 3 (pink). The additional opening of the nucleotide helix that also occurs at that stage is not shown. (B) Close-up view of the BFA-binding site [adapted from [6] with permission (http://www.nature.com/)]. Residues from the Sec7 domain (cyan) whose sequence is critical for the sensitivity to BFA are shown.

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**Interfacial inhibition by BFA of Sec7-stimulated Arf activation**

The above structural studies also uncovered how BFA, a popular inhibitor of membrane traffic at the Golgi [9,15], blocks Arf activation [6,10]. BFA binds at the interface between Arf–GDP and the Sec7 domain in the low-affinity complex that initiates the exchange reaction, freezing it before it has undergone the membrane-securing conformational change that precedes dissociation of GDP. In the cell, BFA is thus likely to act by the titration of BFA-sensitive ArfGEFs, which are in lower abundance than their Arf substrates, resulting in its dominant negative effect. The crystal structures show that the drug interacts with both the small G-protein and the GEF, and is essentially buried at their interface in a hydrophobic cavity (Figure 1B). This cavity is proposed to open in the course of the normal exchange reaction, rather than to be induced by the drug, and to be necessary for the structural dynamics of the exchange reaction [6]. Remarkably, the binding site does not exist in the unbound proteins and requires that both partners are engaged. Thus, BFA inhibits Arf activation by stabilizing rather than blocking the Arf–Sec7 interaction, and it does so by targeting the transient, low-affinity complex that initiates the reaction rather than the high-affinity intermediate that results from nucleotide dissociation.

Remarkably, BFA is also efficient and selective at inhibiting Arf activation in the cells, since only activation of Arf proteins at the Golgi is blocked, whereas BFA has no effect on Arf proteins at the plasma membrane. On the Sec7 side, a single residue change from Tyr to Phe (which impairs a hydrogen bond with the drug) or from Met to Ile (which reduces the size of the hydrophobic cavity) converts BFA-sensitive into BFA-insensitive ArfGEFs (Figure 1B) [10,16,17] (M. Zeghouf, J.-C. Zeeh, B. Guibert and J. Cherfils, unpublished work). It is, however, less clear why Arf6 is insensitive to BFA (M. Zeghouf, J.-C. Zeeh, B. Guibert and J. Cherfils, unpublished work), since all residues of Arf1 that interact with BFA and the GEF are identical in Arf6 at every intermediate of the exchange reaction. Previously reported differences between Arf1-GDP and Arf6-GDP when bound to GDP [18] may play a yet to be identified role in these properties.
on the flatter surfaces of unbound partners, (ii) energetic conditions are unbalanced during conformational changes, thus generating spots with avidity for establishing new interactions and (iii) both components of a complex are recognized, resulting in an increased specificity; as a corollary, the components of a complex become targets for the drug only when they are engaged in a complex, thus selecting the biological pathway and avoiding non-specific inhibitions. There are a variety of protein–protein complexes critical in human diseases which are likely to feature structural flexibility and/or conformational changes (such as those brought about by activation processes) that may constitute their Achilles heel for small molecules to insert and act as stabilizers. In the present study, we will discuss how the interfacial inhibition paradigm may apply in particular to small G-proteins and their GEFs.

Small G-proteins are essential regulators of multiple signalling cascades and are involved in many pathological conditions such as cancer [20–23], inflammation [24], vascular diseases [25], mental retardation [26] and bacterial infections [27]. In a number of cases, small G-proteins are up-regulated due to either overexpression [20,23], activating mutations [22], or by abnormal cellular [28–30] or bacterial [27] GEF activity, or are specifically turned on in pathways that are critical for the onset or progression of the disease [20]. Remarkably, in most cases, GEFs outnumber their small G-protein substrates, suggesting that they may be responsible not only for collecting spatiotemporal activation signals and converting them into a biochemical action (the stimulation of GDP/GTP exchange), but also for channelling the signal into specific downstream pathways [31] (Figure 2). Their contribution to the specificity of signalling is suggested by the GEF-dependent activation of distinct pathways [32], the association of GEF and effectors in multiprotein scaffolds [31,33], or, in the case of Arf1 at the Golgi, by a correlation of the localization of particular ArfGEFs with that of specific effectors [34]. Altogether, this points to GEFs as attractive candidates for the inhibition of small G-protein pathways that are up-regulated or detrimental in diseases. Despite a growing interest in GEFs as therapeutic targets [30,35], there are so far only two inhibitors besides BFA that block the activation of a small G-protein by its GEFs, both targeting Rho/Rac/cdc42 pathways. One targets the RhoGEF Trio, and was identified by a genetic screen of peptide aptamers in yeast [36]; the other targets Rac1 and blocks its activation by its GEFs, and was selected using in silico structure-based screening [37].

The inhibitory properties of BFA validate the concept that small G-proteins can be inhibited in an efficient way by targeting their GEFs, and that an interfacial inhibitor, by modifying the kinetics of the exchange reaction, stalls the downstream events to a sufficient extent as to yield cellular inhibition. The fact that GEFs and small G-proteins both form families of related proteins, for which exquisite specificity is likely to be required, argues in favour of the added specificity expected from interfacial inhibitors. We thus propose interfacial inhibition as an appropriate strategy applicable to the discovery of specific inhibitors of the activation of small G-proteins by their GEFs.

<table>
<thead>
<tr>
<th>Arf protein</th>
<th>Pathogen/disease</th>
<th>Role in disease</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Not known</td>
<td>Vibrio cholerae</td>
<td>Activation of cholera toxin by Arf-GTP</td>
<td>[46,47]</td>
</tr>
<tr>
<td>Not known</td>
<td>Legionella pneumophila</td>
<td>Bacterial ArfGEF secreted in host cytosol</td>
<td>[39]</td>
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<tr>
<td>Arf6</td>
<td>Chlamydia</td>
<td>Recruitment and activation at the bacterial invasion site</td>
<td>[38]</td>
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<tr>
<td>Arf1</td>
<td>HIV</td>
<td>Regulation of Nef-induced CD4 degradation through direct interaction with Nef</td>
<td>[40]</td>
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<tr>
<td>Arf3, Arf5</td>
<td>Poliovirus</td>
<td>Recruited to perinuclear membranes possibly where viral RNA replication occurs</td>
<td>[41]</td>
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<tr>
<td>Not known</td>
<td>Malformation of cerebral cortex (microcephaly, periventricular heterotopia)</td>
<td>Mutation in ArfGEF (BIG2)</td>
<td>[45]</td>
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<tr>
<td>Arf6</td>
<td>Cancer</td>
<td>Activation required for invasion and angiogenesis</td>
<td>[42-44]</td>
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Conclusion and perspective: inhibition of ArfGEFs in diseases

The BFA interfacial model and the structural snapshots of the activation mechanism make Arf proteins and their GEFs an attractive system for the interfacial inhibition strategy. Besides their original identification as activators of cholera toxin [46,47], involvement of Arf proteins and their activation in diseases have recently increasingly been reported (Table 1). Strikingly, Arf-controlled pathways appear to be hijacked by a variety of pathogens. Several intracellular bacteria activate Arf to facilitate their uptake by host cells, which in Chlamidia involves the recruitment of Arf6 and its activation of phosphatidylinositol kinase [38], and in Legionella is mediated by injection of a Sec7-like protein [39]. Arf proteins may also be diverted by viruses, including HIV that may use Nef to bypass the cellular pathway to recruit Arf-GTP to endosomes [40], and polioviruses that translocate several Arf to membranes in a BFA-sensitive manner [41]. Through its functions in co-ordinating membrane traffic and cytoskeletal dynamics [3] Arf6 has also recently focused interest in cancer cells on its role in invasion [42,43] and angiogenesis [44]. The crystal structures of Arf/Sec7 reaction intermediates provide a basis to identify novel binding sites for specific intercalation of, and inhibition by, a small molecule using in silico screening approaches. Structural information for the other small G-proteins and their GEFs is at the moment available only for the nucleotide-free complex, but these proteins are predicted to undergo conformational transitions with similarities to those of Arf and Sec7 and should have appropriate features for interfacial inhibition.

This work was supported by the Association pour la Recherche contre le Cancer and by ACI programs of the French Ministère de la Recherche.

References


Received 22 June 2005