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An Arf1 GTPase mutant with different responses to GEF inhibitors

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ABSTRACT

Guanine nucleotide exchange factors (GEFs) stimulate the activation of small GTP-binding proteins (GTPases). Establishing their specificity is a challenging issue, in which chemical genetics are rapidly gaining interest. We report a mutation in the Arf1 GTPase, K38A, which differentially alters its sensitivity to GEF inhibitors. The mutation renders Arf1 insensitive to LM11, a GEF inhibitor that we previously discovered by structure-based screening. In contrast, full inhibition by the natural compound Brefeldin A (BFA) is retained. We show that the mutation is otherwise silent towards the biochemical and cellular properties of Arf1, notably its binding to effectors as measured by a novel GEF-protection assay. This is thus the first GTPase mutant with different responses to two classes of inhibitors, and a novel tool to analyze Arf and ArfGEF specificity and functions *in vitro* and in cells.

Guanine nucleotide exchange factors (GEFs) stimulate the conversion of small GTP-binding proteins (GTPase) from their inactive, GDP-bound form to their active, GTP-bound form. Although each GTPase family possesses its specific family of GEFs, characterized by a conserved catalytic domain, the mechanism of GDP/GTP exchange is remarkably conserved (reviewed in [1,2]). All GEFs bind to the inactive GTPase–GDP complex, from which they stimulate GDP dissociation by forming a nucleotide-free GTPase/GEF complex. Entry of GTP then promotes the active conformation of the GTPase, which can bind to its effectors. In each GEF family, the conserved catalytic domain is in general active *in vitro* and in cells on more than one GTPase target (reviewed in [3,4]). Conversely, the same GTPase can be activated by different GEFs. Establishing the functional specificity of GEFs is therefore a challenging issue, which can be addressed at the biochemical and cellular levels using small molecules that modulate GEF activities. In addition, the growing recognition of the role of GEFs in human diseases makes inhibitors potential leads to therapeutic molecules [2,5]. To date, inhibitors have been screened mostly for two GTPases families, the Rho family, whose members regulate cytoskeleton dynamics, and Arf GTPases, which are major regulators of cellular traffic. Screening designs included *in silico* structure-based screens [6,7] and screens of chemical libraries by using various approaches [8–11]. The enormous impact of the very first known inhibitor of a GEF, the fungus metabolite Brefeldin A (BFA), illustrates the power of this approach (reviewed in [12]). BFA has been instrumental since the early 90's in deciphering the molecular basis of traffic at the Golgi, and it is still widely used to probe the contribution of trafficking events to various cellular functions. It targets the complex between proteins of the Arf family and the catalytic Sec7 domain of some of their GEFs, which it stabilizes in an abortive Arf–GDP–Sec7 complex [13–16].

With the aim of inhibiting the BFA-insensitive ArfGEF ARNO, we recently used an *in silico* structure-based screen to identify a new inhibitor, LM11 [7]. LM11 inhibits ARNO by a non-competitive mechanism, in which the inhibitor binds to both Arf–GDP and the Arf–GDP/GEF complex. Here, we seeked to identify a mutation in Arf1 that would convert it into an LM11-insensitive version. We report the characterization of such a mutation, K38A, which will allow to analyze Arf and ArfGEF specificities and functions.

Material and methods

Proteins expression and purification. Full length Arf1 cloned into pCMS-EGFP for expression of an N-terminal GFP-tagged protein was a kind gift from C. Jackson (LEBS, Gif-sur-Yvette). The K38A mutation was introduced using the QuikChange™ site-directed mutagenesis kit. The Sec7 domain of human ARNO, either carrying the wild type sequence or the 4 BFA-sensitizing mutations ($ARNO^{4M}$) was expressed and purified as in [16]. The Arf-binding

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domain of ARHGAP21 (ArfBD) was a kind gift of J. Ménétrey and P. Chavrier (Institut Currie, Paris). The K38A mutation was introduced in Δ17Arf1 using the QuikChange™ site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). Δ17Arf1 and Δ17Arf1-^{K38A} were purified essentially as described for Δ17Arf1 in [16]. We verified that the proteins were homogeneously loaded with GDP before the exchange experiments. Circular dichroism scans for the GDP-bound forms were recorded between 185 and 260 nm in 50 mM potassium phosphate buffer pH 8 or 7.4, 50 mM NaF, 1 mM $MgCl_2$.

Exchange and inhibition assays. LM11 was obtained from Chembridge (Chembridge Enterprise), Brefeldin A from Fluka. Stock solution of LM11 and BFA were prepared at 20 mM in dimethyl sulfoxide (DMSO) and in ethanol, respectively. Spontaneous and GEF-stimulated GDP/GTP exchange rates were measured by tryptophan fluorescence (λ_{em} = 292 nm; λ_{ex} = 340 nm). Inhibition assays were monitored by GDP/mantGTP exchange using the fluorescence of the *N*-methylanthraniloyl group (λ_{em} = 360 nm; λ_{ex} = 440 nm). All measurements were performed in 50 mM Tris pH 8, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol, at 37 °C, in the presence of 1 μM Arf. Exchange reactions were initiated by the addition of 100 μM GTP or 10 μM mantGTP. The Sec7 domain concentration was varied between 0.01 and 0.5 μM for the specific exchange rates, and was set to 50 nM for the inhibition assays. Fluorescence data were fitted with KaleidaGraph 3.6.

GEF-protection assay. Δ17Arf proteins (1 μM) were first loaded with mGTP (10 μM) by the Sec7 domain of ARNO (0.1 μM) at 37 °C in 800 μl of reaction buffer (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol). After the fluorescence intensity reached a plateau, either the buffer alone or 4 μM of ArfBD were added and left to incubate for 2 min. The reverse exchange reaction (GTP to GDP) was then started by injection of 200 μM GDP. The apparent mGTP/GDP exchange rate was determined by fitting the fluorescence decay to a single exponential curve.

Confocal microscopy. HeLa cells were transfected using Lipofectamine 2000 (Invitrogen), according to the supplier's instructions. After transfection for 24 h, cells were washed twice with PBS and then subjected to different treatments as indicated in the figure legend. HeLa cells were grown, fixed and observed with either GFP or antibodies as described in [7], except that β-COPI was detected with an Alexa 647-conjugated goat anti-mouse antibody (1:500) (Molecular Probes, Leiden, The Netherlands), using a laser line of 633 nm. Serial sections were acquired satisfying the Nyquist criteria for sampling and processed using the ImageJ 1.40 software (http://rsb.info.nih.gov/ij/).

Results

Selection of a candidate mutation in Arf1

LM11 was originally screened to target a pocket at the interface between Arf1–GDP and ARNO in a crystallographic Arf–GDP/ARNO complex, which had been caught in the act of nucleotide exchange by a mutation of the ARNO catalytic site [15] (Fig. 1A). NMR footprinting experiments indicated that LM11 also binds to uncomplexed Arf1–GDP [7]. Displacements of NMR chemical shifts upon interaction of LM11 with Arf–GDP were highest for Lys38, which is located immediately upstream the switch 1, a region that undergoes large conformational changes during the GDP/GTP cycle (reviewed in [17]). Analysis of crystallographic structures of Arf1 bound to GDP (PDB file 1RRG), to a Sec7 domain (PDB file 1R8S) and to an effector (PDB file 2J59), which altogether recapitulate the GDP/GTP cycle, indicated that this residue is exposed at the surface of all these intermediates, and is not engaged in any strong (<3.5 Å) intramolecular or intermolecular interaction.

These characteristics made it a good candidate for a mutation with the potential to impact on the sensitivity of Arf1 to the inhibitor without impairing its normal functions. We introduced the K38A mutation in human Δ17Arf1, a truncated version of Arf1 that is convenient for kinetics experiments in solution (referred to hereafter as Δ17Arf1^{K38A}). The Δ17Arf1$^{\mathbf{K38A}}$ protein could be expressed with high yields in bacteria and be purified to homogeneity. The circular dichroism spectra of the wild type and mutant proteins are very similar, which indicates that Δ17Arf1$^{\mathbf{K38A}}$ is properly folded (Fig. 1B).

Δ17Arf1^{K38A} is inhibited by BFA but not by LM11

We first analyzed whether Δ17Arf1^{K38A} remained responsive to BFA. We used ARNO^{4M}, an ARNO construct that carries 4 mutations, which render it sensitive to BFA [16]. We controlled that ARNO^{4M} effectively activated Arf1^{K38A}, as confirmed by its specific activity of $3.2 \pm 0.04\ 10^5\ M^{-1}\ s^{-1}$, which was similar to that for Δ17Arf1^{WT} [16]. Δ17Arf1^{K38A} remained highly responsive to BFA, showing only a modest increase of its apparent inhibition constant (Kiapp) from 16 ± 2 μM for Δ17Arf1^{WT} to 32 ± 6 μM for Δ17Arf1-^{K38A} (Fig. 2A). In contrast, whereas Δ17Arf1^{WT} was inhibited by LM11 with a Kiapp of 78 ± 20 μM, Δ17Arf1^{K38A} was completely

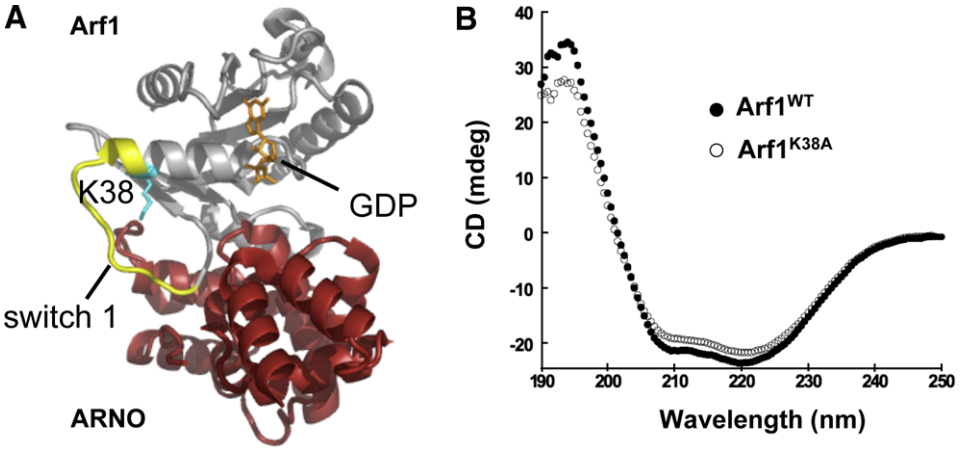

Fig. 1. (A) Structure of the Arf1–GDP/ARNO^{E156K} complex, showing Arf1 in grey with the switch 1 in yellow, and the Sec7 domain of ARNO in red. K38 is shown in cyan. (B) Superposition of the circular dichroism scans for Δ17Arf1^{wt} (black circles) and Δ17Arf1^{K38A} (white circles).

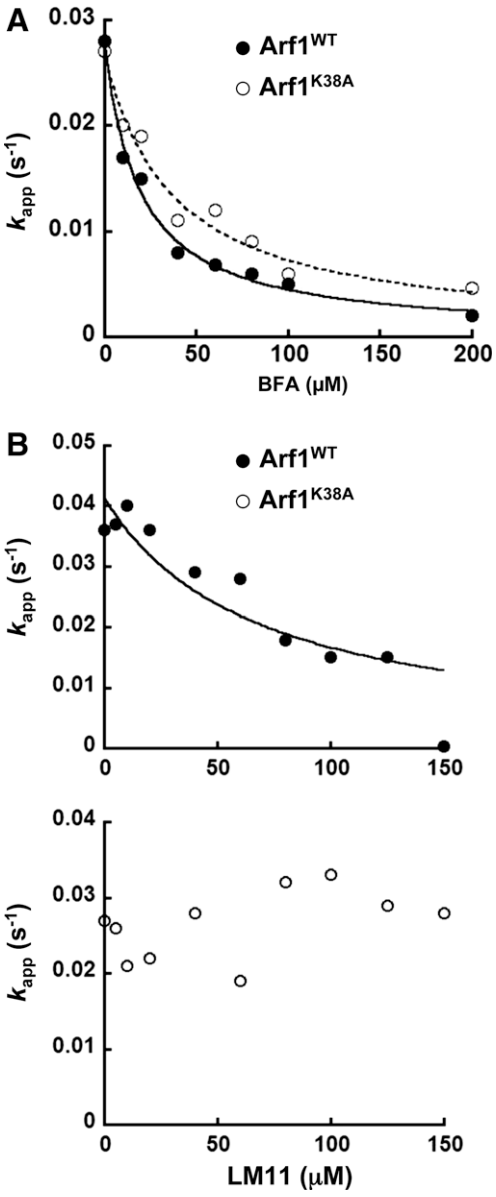

Fig. 2. Inhibition of GDP/mGTP exchange on Δ17Arf1^{wt} (back circles) and Δ17Arf1^{K38A} (white circles). (A) Inhibition by BFA, using the BFA-sensitive ARNO^{4M} construct. (B) Inhibition by LM11, using ARNO^{WT}.

resistant to this inhibitor (Fig. 2B). Consistently, when cells were transfected with GFP–ARF^{K38A}, some perinuclear β-COPI (a subunit of the COPI coatomer, which is a well-characterized marker of the Golgi) structures persisted after LM11 treatment but were almost absent in control cells (Fig. 3C, right panels; see also below). These structures also contained the GFP–ARF^{K38A} protein, which suggests a possible protective effect of the mutant protein. These data identify Arf1^{K38A} as the first GTPase mutant that distinguishes between two different inhibitors.

In vitro and cellular properties of Δ17Arf1^{K38A} are similar to those of Δ17Arf1^{wt}

In order to validate the use of Arf1^{K38A} in subsequent biochemical and cellular experiments, we then investigated for possible defects in the behaviour of the mutant *in vitro* and in cells. We first characterized its nucleotide exchange properties by fluorescence kinetics. We found that Δ17Arf1^{wt} and Δ17Arf1^{K38A} have spontaneous GDP/GTP exchange rates in the same range (respectively $0.0011 \pm 0.0006\ s^{-1}$ and $0.002 \pm 0.0007\ s^{-1}$) and that the catalytic efficiency of ARNO towards Δ17Arf1^{wt} and Δ17Arf1^{K38A} is identical (respectively $2.99 \pm 0.7\ 10^5\ M^{-1}\ s^{-1}$ and $2.99 \pm 0.5\ 10^5\ M^{-1}\ s^{-1}$) (Fig. 3A). This indicates that the K38A mutation is silent for both spontaneous and GEF-stimulated exchange.

We then investigated the ability of Δ17Arf1^{K38A}-GTP to bind to effectors. For that purpose, we devised a GEF-protection fluorescence kinetics assay. This assay takes advantage of the fact that, in general, GEFs catalyze nucleotide exchange in both the forward (GDP → GTP) and reverse (GTP → GDP) directions. This is indeed the case for ARNO and Δ17Arf1 (data not shown). Addition of an effector to the reverse reaction should thus protect Arf–GTP from inactivation by its GEF. We selected for this assay the Arf-binding domain (ArfBD) of human ARHGAP21, a well-characterized Arf1 effector that binds to Arf1–GTP in the micromolar range [18]. Addition of ArfBD to the reverse exchange assay, using Δ17Arf1^{wt} and the Sec7 domain of ARNO, effectively slowed the rate of the reverse exchange reaction by about 2,4 times (Fig. 3B). ArfBD also affected the GTP/GDP exchange rate of Δ17Arf1^{K38A}, although to a lesser extent (reduction of 1.3 times), indicating that the mutant retains the ability to recognize its effector.

We then analyzed the localization of Arf1^{K38A} in HeLa cells. Arf1^{K38A} was expressed as a GFP-fusion protein and its localization was visualized by confocal microscopy. GFP–Arf1^{K38A} stained a compact structure in the perinuclear region, where it colocalized with β-COPI (Fig. 3C). Thus, GFP–Arf1^{K38A} retains the cellular localization of wild-type Arf1 and its expression does not affect the Golgi structure. Altogether, these results indicate that Arf1^{K38A} behaves essentially as wild-type Arf1 *in vitro* and in cells.

Discussion

The LM11 inhibitor was originally screened *in silico*, by targetting a pocket at the interface between the Sec7 domain of ARNO and the switch 1 of Arf1. We showed previously that LM11 binds to both Arf1–GDP and the Arf1–GDP/ARNO complex, and that mutations in ARNO near the interfacial groove reduced the inhibitory activity. In order to refine the structure–activity relationship of LM11, we now show that a single mutation near the switch 1 is sufficient to abolish the sensitivity of Arf1 to LM11 *in vitro*. Together with the high NMR chemical shift displacement observed for K38 [7], these data strongly suggest that K38, hence the switch 1 of Arf1, interacts directly with the inhibitor. Thus, the experimental data on both ARNO and Arf1 are fully consistent with the original screening hypothesis. While NMR HSQC data indicated perturbation at the peptidic amide, which can be indirect, the mutation data now strongly suggest that the side chain of the lysine is actually involved in the interaction. Whether this occurs through an interaction of its aliphatic chain, or by engaging in polar interaction, will require the analysis of LM11 derivatives.

Currently, 3 cell-active ArfGEFs inhibitors have been described: BFA, SecinH3 [10] and LM11. Remarkably, these inhibitors have distinct patterns of activities. BFA has a dual specificity for ArfGEFs and Arfs: it inhibits only the subset of ArfGEFs that carry a tyrosine residue in the toxin-binding site, whose hydroxyl interacts directly with the compound through a hydrogen bond [15,16], and it discriminates also between the closely related Arf1 and Arf6 isoforms [16]. SecinH3 inhibits the ARNO/cytohesin subgroup [10], which BFA does not. LM11 displays another pattern of specificity: it inhibits ARNO like SecinH3 and yet it discriminates between Arf1 and Arf6 like BFA. Mutants that modulate these sensitivities extend the potential of these inhibitors. This is exemplified by mutations that convert BFA-sensitive ArfGEFs into BFA-insensitive versions, which were complementary to BFA itself to study molecular mechanisms of transport in organisms [13,19]. Our new Arf1 mutant

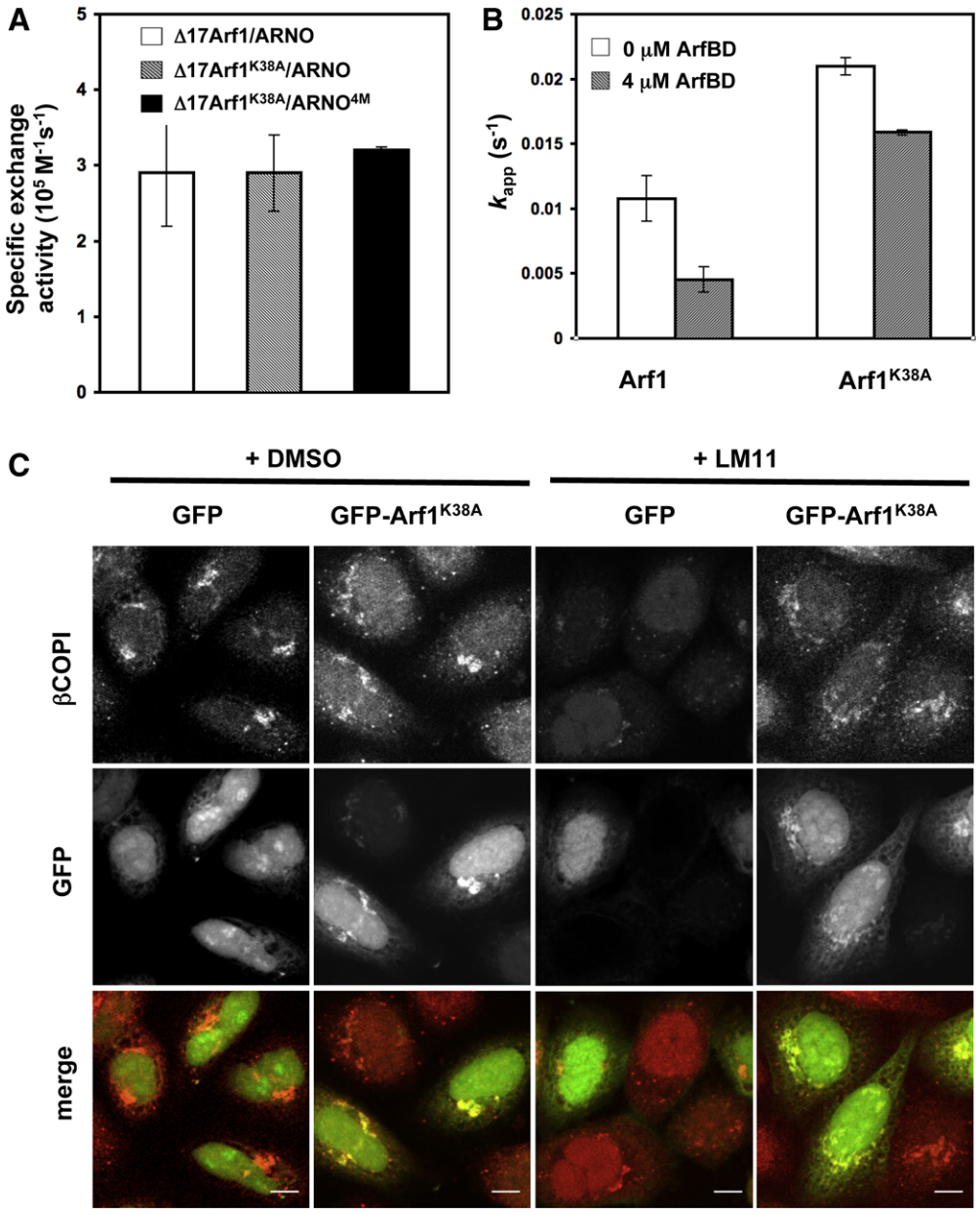

Fig. 3. (A) ARNO-stimulated specific exchange activities for $\Delta17Arf1^{wt}$ and $\Delta17Arf1^{K38A}$. (B) GEF protection assay for $\Delta17Arf1^{wt}$ and $\Delta17Arf1^{K38A}$. The reverse (mGTP/GDP) exchange rates were measured in the presence or absence of the ArfBD effector (see methods). (C) GFP–$Arf1^{K38A}$ colocalizes with β-COPI in HeLa cells as visualized by confocal microscopy. Cells were transfected with GFP-$Arf1^{K38A}$ or GFP alone and treated with either 0.5% DMSO or 100 μM of LM11 for 2 h as indicated on the top. Bar, 8 μm.

now allows to convert an inhibitor-sensitive GTPase into an insensitive one, and it is, to our knowledge, the first report of a GTPase with differential responses to two classes of small molecular weight inhibitors. Taken together, inhibitors and well-characterized mutants should provide a versatile toolbox for analyzing the functional specificity of Arf proteins and GEFs in cells.

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