**Heterotrimeric G Protein Independent Signaling of a G Protein-Coupled Receptor: Direct Binding of ARNO/Cytohesin-2 to the Carboxyl Terminus of the A2A-Adenosine Receptor is Necessary for Sustained Activation of the ERK/MAP-Kinase Pathway**

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Running Title: ARNO/cytohesin-2 in A2A-receptor signaling

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The A2A-adenosine receptor is a prototypical Gs-coupled receptor, but it also signals – e.g. to MAP-kinase (mitogen-activated protein) – via a pathway that is independent of heterotrimeric G proteins. Truncation of the carboxyl terminus affects the strength of the signal through these alternative pathways. In a yeast two-hybrid interaction hunt, we screened a human brain library for proteins that bound to the juxtamembrane portion of the carboxyl terminus of the A2A-receptor. This approach identified ARNO/cytohesin-2 – a nucleotide exchange factor for the small (monomeric) G proteins of the ADP-ribosylation factor (Arf) family - as a potential interaction partner. We confirmed a direct interaction by mutual pull-down (of fusion proteins expressed in bacteria) and by immunoprecipitation of the proteins expressed in mammalian cells. In order to circumvent the long term toxicity associated with overexpression of ARNO/cytohesin-2, we created stable cell lines that stably expressed the A2A-receptor and where ARNO/cytohesin-2 or the dominant negative version E156K-ARNO/cytohesin-2 was inducible by mifepristone. Cyclic AMP accumulation induced by an A2A-specific agonist was neither altered by ARNO/cytohesin-2 nor by the dominant negative version. This was also true for agonist-induced desensitization. In contrast, expression of dominant negative E156K-ARNO/cytohesin-2 and of dominant negative T27N-Arf 6 abrogated the sustained phase of MAP-kinase stimulation induced by the A2A-receptor. We therefore conclude that ARNO/cytohesin-2 is required to support the alternative – heterotrimeric G protein-independent - signaling pathway of A2A-receptor, which is stimulation of MAP-kinase.

Over the last decade, it has been increasingly accepted that G protein-coupled receptors also bind regulatory proteins other than G proteins, arrestins and G protein-coupled kinases, which are involved in effector regulation and desensitization, respectively (1). These accessory proteins include components of signaling cascades and bind to the carboxyl termini of various G protein-coupled receptors (2). The A2A-adenosine receptor has an unusually long intracellular carboxyl terminal tail, 122 amino acids in man, when compared for instance to 34 residues in the carboxyl terminus of the A1-adenosine receptor. Circumstantial evidence suggests that accessory proteins bind to the carboxyl terminus of the A2A-receptor (3). A2A-receptors can activate mitogen activated protein (MAP) kinase by a Gαs-independent signaling pathway; this can be seen both in endothelial cells where the receptor is endogenously expressed (4) and upon heterologous expression in HEK293 cells (5). Truncation of the carboxyl terminus does not
impair the ability of the A2A-adenosine receptor to stimulate MAP-kinase but blunts stimulation of cAMP accumulation. In addition, full length and truncated receptors differ in their constitutive (agonist-independent) activity; this difference is only seen in intact cells and is lost upon membrane preparation suggesting the loss of one or several interacting proteins (6). These findings are consistent with earlier experiments where the A2A-receptor was found to be tightly coupled to its cognate G protein. Tryptic cleavage of the receptor, which presumably released the carboxyl terminus, abrogated the tight complex and restored guanine nucleotide sensitivity to the ternary complex of agonist, receptor and G protein (7,8). Taken together, these observations suggest an important role for the carboxyl terminus in signaling by the A2A-receptor. In the present paper we identify ARNO/cytohesin-2, an exchange factor for low molecular weight G proteins of the ADP-ribosylation factor (Arf) family, as a direct interaction partner of the C-terminus of the A2A-receptor. The presence of ARNO/cytohesin-2 is required to support the alternative signaling to MAP-kinase, because dominant negative ARNO/cytohesin-2 efficiently suppresses the sustained phase of receptor dependent MAP-kinase phosphorylation.

Experimental Procedures

Materials and reagents: Adenosine deaminase and Complete® protease inhibitor tablets were from Roche Molecular Biocals (Mannheim, Germany). CGS21680 and [3H]JM241385 (specific activity 27.4 Ci/mmol) were from Tocris Cookson Ltd. (Bristol, UK). XAC was obtained from Research Biocals (Natick, MA, USA). Materials required for SDS-PAGE were from BioRad (Richmond, CA, USA). Fetal calf serum was from PAA Laboratories (Linz, Austria), Dulbecco’s modified Eagle medium (DMEM), non-essential amino acids, β-mercaptoethanol, gentamicin, G418 (geneticin), Lipofectamine and Lipofectamine Plus reagent were obtained from GIBCO-BRL (Grand Island, NY, USA). Zeocin and hygromycin were from Eubio (Vienna, Austria). Brefeldin A, cyclic adenosine 3’,5’ monophosphate (cAMP), forskolin, L-glutamine, streptomycin, triton X-100, PMSF, anti-flag-M2-affinity gel and peroxidase-conjugated anti-flag monoclonal antibody, monoclonal anti-HA peroxidase-conjugated antibody were purchased from Sigma Co. (St. Louis, MO, USA). Rabbit anti-GFP Living colors A.v. peptide antibody were purchased from Clontech Laboratories, Inc (Mountain View, CA, USA). Horseradish peroxidase-conjugated anti-mouse- and anti-rabbit immunoglobulin antibodies were from Amersham Life Science (Buckinghamshire, UK.). The immunoreactive bands on nitrocellulose blots were detected by chemiluminescence using SuperSignal chemiluminescence substrate from Pierce (Rockford, IL, USA). GSH-Sepharose and pGEX-5X-1 were from Amersham-Pharmacia (Freiburg, Germany); pMAL vector and amylose resin for expression and purification of maltose binding protein (MBP) were purchased from New England Biolabs, Inc (Beverly, MA, USA). The Micro BCA® protein assay reagent kit was from Pierce (Rockford, IL, USA). Buffers and salts were from Merck (Darmstadt, Germany). [3H]Adenine was from DuPont NEN (Boston, MA, USA). Centrifuge tubes and tissue culture plates were from Greiner (Vienna, Austria) and from Cornig Costar (Acton, MA, USA). Plasmid preparation kits were from Qiagen (Hilden, Germany).

DNA constructs. A DNA fragment encoding the C-terminal part of A2A receptor was amplified by PCR from pcDNA3-A2AR vector (6). After restriction digestion the PCR fragment was inserted into pEG202 for the yeast two-hybrid screen (pEG202-A2ARct) and into pMAL-cri for in-vitro pulldown assays (MBP-A2ARct). An identical strategy was employed for the generation of the carboxy terminally truncated versions A2AR(311) and A2AR(360) (6). For epitope tagging of the A2A receptor, the sequence of the receptor, encompassing the coding region, was ligated into HindIII/Sall-digested pCMV-Tag 2B
vector, linking the A2AR in frame to the N-terminus of the flag-epitope. The cDNA constructs for ARNO/cytohesin-2 and the dominant negative variant were a kind gift of P. J. Cullen (University of Bristol, UK); they were amplified by PCR and subcloned into the pGEX vector. A plasmid encoding for 84 amino acid fragment of protein 1,4-galactosyl-transferase fused to YFP to specifically label the Golgi apparatus was purchased from Clontech Laboratories, Inc (Mountain View, CA, USA). The cDNA construct for the dominant negative version of Arf 6 was a kind gift of Dr. J. G. Donaldson (NIH, USA).

Yeast two-hybrid interaction hunt. Yeast two-hybrid analysis was performed using the MATCHMAKER yeast two-hybrid system (Clontech Laboratories, Inc). In this system, yeast strain EGY48 (MATα trp1 his3 ura3 leu2 6 LexAop-LEU2) was used for transformation of bait, prey and reporter plasmids: pEG202, pJG4-5 and pSH18-34, respectively. EGY48 containing the lacZ reporter plasmid (pSH18-34) and the A2ARcT bait plasmid (pEG202-A2ARcT) was transformed with prey plasmids bearing human brain cDNA library (Clontech Laboratories, Inc). Selection for colonies that express interacting proteins were done by using the auxotrophy markers according to the manufacturer’s instructions. To verify the two-hybrid interactions, positive transformants grown on selection medium were assayed directly in situ for β-galactosidase activity assay by briefly exposing the plates to chloroform and subsequent incubation with 100 µM X-gal in the medium as a substrate.

Cell culture, creation of stable cell lines, radioligand binding assay, cellular cAMP accumulation, MAP-kinase stimulation. HEK293 cells were maintained in Dulbecco’s modified Eagle medium (DMEM.) at 5% CO2, 95% air and 37°C. Culture media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, β-mercaptoethanol and non-essential amino acids. Cells were transfected by using LipofectAMINE Plus™. Because forced transient expression of ARNO/cytohesin-2 altered cell morphology and reduced cell viability, we transfected HEK293 cells with a plasmid encoding GAL4-DBD/hPR-LBD/p65-AD, a fusion protein that comprises the DNA-binding domain of GAL4, the ligand binding domain of the progesterone receptor and the activation domain of the human p65 subunit of NF-κB; this protein is activated by mifepristone (9). The selection marker was hygromycin. Stable cell clones were selected and subjected to a second transfection with a plasmid engineered to encode ARNO/cytohesin-2 or dominant negative E156K-ARNO/cytohesin-2 under the control of 6 GAL4-responsive upstream activating sequences (UAS). Stably transfected cells were selected in the presence of zeocin. Two cell lines with a robust induction of ARNO/cytohesin-2 and its dominant negative version were selected and transfected with a third plasmid encoding for the flag-tagged A2A-receptor, the selection marker being geneticin. Cell lines were selected that expressed equally high levels of the receptor. Media for the culture of stably transfected cells were supplemented with 0.2 mg/mL geneticin (G418), 125 mg/L zeocin and 50 mg/L hygromycin in order to maintain the selection pressure. Expression of ARNO/cytohesin-2 or of E156K-ARNO/cytohesin-2 was induced by the addition of 10 nM mifepristone and monitored by immunoblotting of cellular lysates with an antiserum against ARNO/cytohesin-2. Membranes were prepared from uninduced and induced cells and binding of the antagonist radioligand [3H]ZM241385 was determined as described (6). The conditions for stimulation of MAP-kinase are also outlined therein (6). Immunoreactive bands were quantified using the densitometric quantification program Scion Image by Scion Corporation (Frederick, MD, USA). The cellular ATP pool was metabolically prelabeled by incubating cells (10⁷/well of a 6-well dish) for 16 h with 0.2 µCi [3H]adenine. Assay
conditions for agonist-induced cAMP accumulation were as in ref. 10.

**Purification of fusion proteins and in vitro pull-down assays.** E. coli (BL21DE3) were transformed with plasmids encoding fusion protein constructs; bacteria were grown at 37°C to an O.D.\textsubscript{600} of ~0.6. Protein expression was induced by addition of 0.5 mM IPTG for 2.5 h. GST-ARNO/cytohesin-2 and GST-PH fusion proteins were purified by affinity chromatography on GSH-sepharose 4 fast flow (Amersham Biosciences) as in ref. (11) by using 15 mM GSH to elute the fusion protein; MBP-A\textsubscript{2A}R\textsubscript{Ct} fusion protein was purified in an analogous way by amylose resin chromatography and by using 20 mM maltose to release the immobilized fusion protein. For pull-down assays the indicated amounts of proteins were incubated in the presence of GSH-sepharose (20 µL a 50 % slurry) at 4°C overnight. After subsequent washing with GST buffer (25 mM HEPES pH 8.0, 1% Triton X-100, 150 mM NaCl, 1mM EDTA, 2mM MgCl\textsubscript{2}), protein complexes were released by yeast denaturation in SDS sample buffer and were subjected to SDS polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membranes. Immunoreactive bands were detected with polyclonal antisera against GST and MBP and visualized by enhanced chemiluminescence.

**Fluorescence microscopy.** Confocal microscopy was performed under oil immersion using a laser scan microscope (Zeiss Axiovert LSM510). FRET (fluorescence energy resonance transfer) microscopy was done as outlined in detail previously (12) with a Zeiss Axiovert 200M inverted epifluorescence microscope equipped with a CoolSNAP fx cooled CCD camera (Photometrics, Roper Scientific, Tucson, AZ). In brief, cells were seed on coverslips and co-transfected with plasmids encoding CFP-tagged ARNO and YFP-tagged A\textsubscript{2A}-receptor. Twenty four hours after transfection, the coverslips were mounted in the microscope chamber. Images of cells with CFP-tagged ARNO and YFP-tagged A\textsubscript{2A}-receptor were captured through corresponding filter channels. To measure donor recovery after acceptor photobleaching (DRAP), we acquired a donor (CFP) image before and after photobleaching using the YFP setting for 90 s (excitation 500 nm, dichroic mirror 525 nm, and emission 535 nm) to calculate a ratio image. Fluorescence images were analysed using the MetaSeries software MetaFluor and MetaMorph (release 4.6, Universal Imaging Corp., Downingtown, PA, USA).

**Immunoprecipitation of the epitope-tagged A\textsubscript{2A}-receptor.** Membranes (0.5 mg) were solubilized in 1 mL ice-cold lysis buffer [50 mM Tris.HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, containing 1% Nonidet P-40, protease inhibitors (Complete, Roche Molecular Biochemicals, Germany)] for 1 hour on ice. After centrifugation at 100,000 × g for 60 min, the supernatant was incubated with anti-Flag M2 affinity matrix (0.1 mL of pre-equilibrated 50% slurry) for 16 h at 4°C; after repeated washing in buffer, bound proteins were released by heat denaturation in SDS-containing sample buffer, subjected to SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with an antiserum directed against ARNO/cytohesin-2 or with the antibody directed against the flag-epitope.

**Results**

The carboxyl terminus of the A\textsubscript{2A}- adenosine receptor (A\textsubscript{2A}R\textsubscript{Ct}) was used as bait in a yeast two-hybrid interaction hunt: a human brain cDNA library was searched for potential interaction partners. The minimum sequence that is required to support efficient MAP-kinase activation by the A\textsubscript{2A}- receptor is a construct that contains the first 22 amino acids of the carboxyl terminus (6). Of the 18 positive clones that were first identified, we selected a prey insert that - upon retransformation - still interacted with truncated versions of the carboxyl terminal tail as strongly as the with the full length C-terminus (Fig. 1A, left panel). The only prey...
insert that fulfilled this criterion contained the pleckstrin-homology (PH) domain of ARNO/cytohesin-2 (ARNOPH); this prey interacted equally well with two mutated versions, which correspond to the removal of 100 and 51 amino acids for carboxyl terminus, \( A_{2A}RC_{x1} \) (290-311) and \( A_{2A}RC_{x1} \) (290-360), respectively. The interaction with both truncated versions induced the same level of \( \beta \)-galactosidase activity as compared to the full length carboxyl terminus (Fig. 1A, left panel). As an internal control we used USP4, a different interactor, which also binds to the \( A_{2A} \)-receptor C-terminus\(^2\); in this case, truncation of the last 50 amino acids greatly reduced the capacity of the bait to induce \( \beta \)-galactosidase activity (Fig. 1A, right panel).

The direct physical interaction between the two proteins was confirmed \textit{in-vitro} by a glutathione-S-transferase (GST) pull-down assay (Fig. 1B). A purified fusion protein comprising the maltose binding protein and the carboxyl terminus of the \( A_{2A} \)-receptor (\( A_{2A}RC_{\text{CT-MBP}} \)) was incubated with GSH-sepharose in the presence of purified GST-full length ARNO/cytohesin-2, GST-PH domain of ARNO/cytohesin-2 or of GST alone. \( A_{2A}RC_{\text{CT-MBP}} \) fusion protein was eluted as a complex with GST-full length ARNO/cytohesin-2 (Fig. 1B, lane 5, 6) and GST-ARNOPH (Fig. 1B, lane 3, 4) but not with GST alone (Fig. 1B, lane 1, 2). These results confirm the findings of the yeast two-hybrid experiments. More importantly, they rule out that the interaction arises from an intermediary protein which binds to both ARNO/cytohesin-2 and the carboxyl terminus of the \( A_{2A} \)-receptor and the ortholog of which may be present in yeast. \( \beta \)- Arrestin, for instance, has been proposed to allow for the recruitment of Arf 6 and ARNO to mediate endocytosis of the \( \beta_2 \)-adrenergic receptor (13). While data in Fig. 1A & B show that the carboxyl terminus can interact with the PH-domain of ARNO/cytohesin-2, it does not prove that the receptor binds ARNO/cytohesin-2 \textit{in vivo}. In order to visualize the distribution of the two proteins in living cells, we tagged the C-terminus of the \( A_{2A} \)-adenosine receptor and the N-terminus of the ARNO/cytohesin-2 with spectral variants of the green fluorescent protein, yellow (YFP) and cyan (CFP) fluorescent protein, respectively. Wild-type and YFP-tagged receptors did not differ in their ability to raise cAMP upon agonist stimulation, i.e. the agonist potency and efficacy were comparable (data not shown). Thus, YFP-\( A_{2A} \)-adenosine receptors were functionally indistinguishable from their wild-type counterparts. When transiently expressed in human embryonic kidney (HEK-293) cells YFP-tagged \( A_{2A} \)-adenosine receptors were localized at the cell surface and to some extent in intracellular vesicles (Fig. 2A, left panel). In contrast, confocal imaging of CFP-tagged ARNO/cytohesin-2 showed a distribution throughout the cytoplasm (Fig. 2A, middle panel). Interestingly, in cells expressing high levels of CFP-ARNO/cytohesin-2, there appeared to be more YFP-tagged \( A_{2A} \)-adenosine receptors in the intracellular vesicular compartment (Fig. 2A, cell in the middle and cell on the right). If the two images were overlayed, there was evidence for widespread co-localization of the two proteins at the plasma membrane (cf. green color in Fig. 2A, right panel). Overlays are indicative of a close spatial relationship rather than a proof of a direct interaction between proteins. This can be demonstrated by using fluorescence resonance energy transfer (FRET) microscopy. In order to visualize the interaction of the ARNO/cytohesin-2 and the \( A_{2A} \)-receptor, we applied the FRET method of “donor recovery after acceptor photobleaching” (DRAP-FRET). This method has the advantage that it yields valid results, even if the expression levels of acceptor and donor protein are different. The interaction of a pair of FRET fluorophores within the Foerster distance renders the donor less susceptible to bleaching or enhances donor emission upon acceptor bleaching. Obviously, high levels of illumination lead to a rapid decline of either acceptor or donor fluorescence. In the case of DRAP-FRET, the parameters were set to preserve donor fluorescence while obtaining...
maximum acceptor bleaching (14). In HEK293 cells overexpressing YFP-A2A-adenosine receptors and CFP-ARNO/cytohesin-2, we observed a resonance energy transfer limited to the cell membrane (Fig 2B). Cells with YFP-fluorescence (before bleaching) are shown in the left panel of Fig. 2B to confirm the expression of the A2A-receptor. Cells that only contain YFP-tagged A2A-receptor (but no ARNO/cytohesin-2, marked by * in Fig. 2B) do not show any FRET. In cells which express both, YFP-tagged A2A-receptor and CFP-tagged ARNO/cytohesin-2, the increase in donor fluorescence upon acceptor bleaching is confined to the membrane area (cf. arrows in right panel, Fig. 2B). Thus resonance energy transfer only takes place at the plasma membrane although the fluorescence signal for ARNO/cytohesin-2 is evenly distributed throughout the cytoplasm (cf. middle panel, Fig. 2B) and although there is significant intracellular accumulation of A2A-receptors in cells that express ARNO/cytohesin-2 (cf. left panel, Fig. 2B). These experiments show that the A2A-receptor and ARNO/cytohesin-2 do not only interact when expressed as fusion proteins in yeast or in bacteria but also interact when co-expressed in mammalian cells. These findings also suggest that this interaction takes place at the inner leaflet of the plasma membrane.

However, in most of the experiments uncontrolled overexpression of ARNO/cytohesin-2 resulted in strong phenotypic effects on HEK293 cell morphology and promoted cell rounding (Fig. 2B) and subsequently cell detachment. On the other hand endogenous expression of ARNO/cytohesin-2 in HEK293 cells is very low and barely detectable by immunoblots with available antisera/antibodies (Fig 3A, cf. lanes 1, 4 & 5 and lanes 2 & 3). We therefore generated a HEK293 cell line which stably expressed a flag-tagged version of the A2A-receptor and in which expression of ARNO/cytohesin-2 was inducible (wt-ARN-A2A cells). A corresponding cell line was also generated for the dominant negative version E156K-ARN-A2A cells); the point mutation E156K renders ARNO/cytohesin-2 catalytically inactive (15). In these cell lines the synthetic promoter of ARNO/cytohesin-2 is under the control of a GAL4-DBD/hPR-LBD/p65-AD regulatory fusion protein, which is activated by binding of mifepristone. Mifepristone drives the expression of ARNO/cytohesin-2 as well as the expression of GAL4-DBD/hPR-LBD/p65-AD through an auto regulatory feedback loop (9). Stable levels of ARNO/cytohesin-2 or of E156K-ARNO/cytohesin-2 were detected between 8 and 48 hours or 8 to 30 hours after addition of mifepristone, respectively. We therefore performed all following experiments 8 hours after induction of wild type ARNO/cytohesin-2 and dominant negative ARNO/cytohesin-2 and compared the response of these cells to cells that had not been treated with mifepristone. Under these conditions, the morphology of the cells was unchanged and the levels of A2A-receptor expression were not affected. Finally, we verified that mifepristone did not affect A2A-receptor mediated cAMP accumulation and MAP-kinase stimulation in cells that were devoid of a mifepristone-inducible ARNO (data not shown).

We incubated cell extracts prepared from wt-ARN-A2A cells with anti-flag antibodies immobilized on sepharose beads. The eluate was analyzed by immunoblotting for ARNO/cytohesin-2: immunoreactive material was recovered with the antibody directed against the flag-tagged A2A-receptor, if cells had been induced to express ARNO/cytohesin-2 (Fig. 3C, lane 2). A faint band is seen in the eluate of cells that had not been treated with mifepristone(Fig. 3C, lane 3). Because this band comigrates with overexpressed ARNO, it is likely to represent coimmunoprecipitated endogenous ARNO/cytohesin-2. As a control of specificity, we used extracts from cells, which overexpress ARNO/cytohesin-2 but do not express the A2A-receptor; there is no immunoreactivity for ARNO/cytohesin-2 in this lane (Fig 3C, lane 4). These findings corroborate the DRAP-FRET experiments that the A2A-receptor and ARNO/cytohesin-2
are indeed able to form complexes in mammalian cells.

ARNO/cytohesin-2 is thought to act as the guanine-nucleotide exchange factor of Arf 6 (16), a small G protein that affects cell mobility by remodelling of the cortical actin (17). In addition, in polarized cells, Arf 6 can specify the distribution of G protein-coupled receptors to distinct membrane compartments (18). Finally, in the LH/HCG-receptor, another Gs-coupled receptor, ARNO/cytohesin-2 is thought to promote receptor desensitization (19,20). We therefore determined the effect of ARNO/cytohesin-2 on A2A-receptor signaling. For these experiments, we selected two ARN-A2A cell lines that had reasonably comparable levels of receptor expression, that is the Bmax values were 10.9 ± 1.6 and 12.5 ± 1.7 pmol/mg protein for cell lines with inducible ARNO/cytohesin-2 and the dominant negative mutant, respectively (Fig. 4A & B). Neither the expression levels nor the binding affinity for the A2A-antagonist radioligand [3H]ZM241385 were affected by the induction of ARNO/cytohesin-2 (Fig. 4A) or of the dominant negative version (Fig. 4B). To investigate effects on GoS-dependent activation of adenylyl cyclase we determined cAMP levels after induction of wild type ARNO/cytohesin-2 and of dominant negative ARNO/cytohesin-2 and compared the response of these cells to cells that had not been treated with mifepristone. The A2A-selective agonist CGS 21680 elicited cAMP accumulation with a potency that was comparable within experimental error in the two cell lines. Induction of wild-type ARNO/cytohesin-2 (Fig. 4C) or of the dominant negative mutant (Fig. 4D) did not affect the concentration-response curve for CGS 21680 to any significant extent (EC50 = 1.2 ± 0.5 nM and 2.3 ± 0.7 nM in wtARN-A2A and dnARN-A2A cells, respectively). Upon continuous exposure to an agonist, the A2A-adenosine receptor rapidly loses its ability to elevate cAMP levels (21). Because ARNO/cytohesin-2 is thought to regulate receptor desensitization (13,19,20), we also examined the effect of ARNO/cytohesin-2 on cells that had been pretreated with saturating agonist concentrations for two hours at 37°C. Then, CGS21680 was removed by rapid repeated washing and the cells were re-challenged with various concentrations of the agonist. When compared to cells that received only vehicle (Fig. 4E & F), the pre-stimulated cells showed higher basal cAMP levels. This reflects either residual activation or – more likely - adaptive changes of the adenylyl cyclase. However, cAMP levels were further stimulated by addition of the agonist. Again there was no difference between cells over-expressing wild-type ARNO/cytohesin-2 (Fig. 4E) compared to cells over-expressing the dominant negative mutant (Fig. 4F). Based on these observations, we conclude that ARNO/cytohesin-2 is neither involved in the GoS-mediated activation of adenylyl cyclase by the A2A-receptor nor does it play a role in A2A-receptor desensitization.

In addition to stimulation of adenylyl cyclase via Gs, the A2A-adenosine receptor is coupled to activation of MAP-kinase by a Gαs-independent signaling pathway (4,5) that also cannot be accounted for by any other heterotrimeric G protein (6). Hence, we investigated the role of ARNO/cytohesin-2 in this alternative signaling pathway. Cells were incubated with 1 µM CGS 21680 and harvested at the indicated time points. Equal amounts of cells lysates were probed with an antiserum directed against the phosphorylated p42/erk2- and p44/erk1-isoforms of MAP-kinase (Fig. 5, upper panels). Activation of MAP-kinase was observed as a biphasic increase of the phospho-specific immunoreactivity in cells that had not been treated with mifepristone. The A2A-selective agonist CGS 21680 elicited cAMP accumulation with a potency that was comparable within experimental error in the two cell lines. Induction of wild-type ARNO/cytohesin-2 (Fig. 4C) or of the dominant negative mutant (Fig. 4D) did not affect the concentration-response curve for CGS 21680 to any significant extent (EC50 = 1.2 ± 0.5 nM and 2.3 ± 0.7 nM in wtARN-A2A and dnARN-A2A cells, respectively). Upon continuous exposure to an agonist, the A2A-adenosine receptor rapidly loses its ability to elevate cAMP levels (21). Because ARNO/cytohesin-2 is thought to regulate receptor desensitization (13,19,20), we also examined the effect of ARNO/cytohesin-2 on cells that had been pretreated with saturating agonist concentrations for two hours at 37°C. Then, CGS21680 was removed by rapid repeated washing and the cells were re-challenged with various concentrations of the agonist. When compared to cells that received only vehicle (Fig. 4E & F), the pre-stimulated cells showed higher basal cAMP levels. This reflects either residual activation or – more likely - adaptive changes of the adenylyl cyclase. However, cAMP levels were further stimulated by addition of the agonist. Again there was no difference between cells over-expressing wild-type ARNO/cytohesin-2 (Fig. 4E) compared to cells over-expressing the dominant negative mutant (Fig. 4F). Based on these observations, we conclude that ARNO/cytohesin-2 is neither involved in the GoS-mediated activation of adenylyl cyclase by the A2A-receptor nor does it play a role in A2A-receptor desensitization.

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contrast, exchange factors for Arf 6 are resistant to the action of brefeldin A (22,23,24). If endogenous ARNO/cytohesins-2 and Arf 6 were involved in A2A-receptor-dependent stimulation of MAP-kinase, brefeldin A should not affect signaling. This prediction was verified: addition of brefeldin A did not alter the time course of agonist-stimulated MAP-kinase phosphorylation (Fig. 6A & B). The effectiveness of brefeldin A treatment was tested in parallel incubations by visualizing the Golgi apparatus with a specific, fluorescent Golgi-marker (84 amino acid fragment of protein 1,4-galactosyl-transferase fused to YFP); treatment of cells with brefeldin A disrupts anterograde membrane flow through the Golgi and thus results in fragmentation of the Golgi stacks (25). Prior to addition of brefeldin A, the Golgi marker associated fluorescence was concentrated over a structure located in the perinuclear vicinity (Fig. 6C, upper right panel, b; cf. correspond phase contrast in panel a). After 30 and 120 minutes incubation of the cells with 5 µg/ml brefeldin A, the fluorescent signal was diffusely distributed throughout the cytoplasm (Fig. 6C, lower panels, c & d). Thus, the concentration of brefeldin A sufficed to block the action of exchange factors for Arf 1-5. Taken together these experiments provided circumstantial evidence for a role of Arf 6 in A2A-receptor-mediated activation of MAP-kinase. This conjecture was corroborated by co-expressing the receptor and a dominant negative version of Arf 6 (T27N-Arf 6). While present in control cells (Fig. 7A), the second phase of MAP-kinase activation was absent in cells that contained T27N-Arf 6 (Fig. 7B).

Discussion
To the best of our knowledge, the A2A-receptor is the first G protein-coupled receptor that has been shown to interact directly with ARNO/cytohesin-2. Previously, two members of the G protein-coupled receptor family have been found to require ARNO/cytohesin-2 during desensitization, namely the β2-adrenergic receptor (13,26) and the luteinizing hormone/choriogonadotropin- (LH/CG-) receptor (19,20,27). In both instances, the role of ARNO/cytohesin-2 is indirect and conditional on the presence of β-arrestin: ARNO/cytohesin-2 has been proposed to promote binding of β-arrestin to the receptor (19,20,27); alternatively, β-arrestin is thought to serve as a scaffold to allow for activation of Arf 6 by ARNO/cytohesin-2 (13). The present work, however, shows that the A2A-adenosine receptor differs from these two paradigmatic examples because it binds ARNO/cytohesin-2 directly. In addition, direct binding of ARNO/cytohesin-2 apparently provides one of the missing links to the alternative signaling pathway, which the receptor can employ in some cells (e.g. endothelial cells and HEK293 cells), rather than to desensitization.

The current observations also indicate that the endogenous levels of ARNO/cytohesin-2 present in HEK293 cells are sufficient and necessary for sustained activation of the MAP-kinase signaling pathway. It is worth noting that neither dominant negative ARNO nor Arf 6 affected the initial stimulation of MAP-kinase phosphorylation. Thus, at the very least, the observations imply the existence of two, presumably independent signaling pathways to support the typical biphasic MAP-kinase stimulation induced by the A2A-receptor. It is at present unknown, how the interaction of ARNO/cytohesin-2 with the A2A-receptor supports sustained activation of MAP-kinase. However, it is not without precedent: inhibition of ARNO/cytohesin-2 was recently found to down-regulate mitogen-activated protein kinase activation in HeLa cells (28).

Arf 6 is believed to control actin remodeling and vesicle trafficking by activating phospholipase D (29) and phosphatidylinositol-4-phosphate-5-kinase-αI (30), but there is at least one additional effector that links Arf 6 to activation of rac1 (31,32). It is not clear how any of these activities translate into stimulation of MAP-kinase. It has been argued that activation of MAP-kinase requires internalization of the
receptor and that this may be related to sustained stimulation of MAP-kinase. This observation was originally made with the EGF-receptor (33) and subsequently also shown to be true for some G protein-coupled receptors (34). However, blocking endocytosis of the A2A-receptor by dominant negative dynamin K44A does not affect agonist-mediated stimulation of MAP-kinase (6,35). Thus, endocytosis is unlikely a candidate mechanism to explain the action of ARNO/Arf 6 in the signaling pathway linking the A2A-receptor to sustained MAP-kinase stimulation. In fact, we did not observe any change in the desensitization of agonist-induced adenylyl cyclase activation, regardless whether wildtype or dominant negative ARNO/cytohesin-2 was present. Likewise, the sustained phase of MAP-kinase activation has been proposed to depend on activation of Ras in the Golgi stack (36,37). However, it is unlikely that an intact Golgi apparatus is required for the sustained phase of MAP-kinase stimulation by the A2A-receptor: brefeldin A did not alter the time course of A2A-agonist dependent MAP-kinase phosphorylation, although the Golgi stacks were rapidly and effectively dispersed at the concentration employed. Thus, the available data suggest that the action of ARNO/cytohesin-2 and Arf 6 primarily originates at the cell membrane and this was also the site where the A2A-receptor and ARNO/cytohesin-2 were colocalized.

References

Footnotes
* This work was supported by grants from the Austria Science Foundation (FWF) P15306 and P17023 to M.F. and J.Z., respectively.
1Abbreviations used:
ARNO, Arf-nucleotide site opener; Arf, ADP-ribosylation factor; CGS21680, N-ethylcarboxamido-2-[4-(2-carboxyethyl)phenylethyl]adenosine; GST, glutathione transferase; MAP-kinase, mitogen-activated protein kinase (also referred to as erk1/2 extracellular signal regulated kinase-1 and –2); MBP, maltose binding protein; XAC, xanthine amino congener; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ZM241385, 4-(2-[7-amino-2-[2-furyl]1,2,4]triazolo[2,3-a][1,3,5] triazin-5-yl-amino)ethyl)phenol
2T. Milojevic and M. Freissmuth, manuscript in preparation
**Figure Legends**

**Fig. 1. Interaction of the carboxyl terminus of the A2A-adenosine receptor with ARNO/cytohesin-2.**  
*A*, Yeast-two hybrid interaction assay: Yeast strain EGY48 were re-transformed with the bait plasmid pEG202-A2AR<sub>CT</sub>, which encoded the full length carboxyl terminus of the A<sub>2A</sub>-receptor (aa 290 to 411) or plasmids encoding truncated versions thereof and the prey plasmid pJG45-ARNO<sub>PH</sub> (right panel) or – as an internal control - pJG45-USP4 (left panel). Cells were streaked on a galactose-containing plate in duplicates, allowed to grow and then stained for β-galactosidase activity by incubation with X-gal (100 µM) for 2 h. The constitutive and a galactose-inducible controls correspond to yeast cells transformed with a plasmid encoding the lexA binding domain fused to the transactivation domain and with plasmids encoding the regulatory B<sub>α</sub> protein phosphatase 2A fused to lexA and A<sub>α</sub> subunit of protein phosphatase fused to the transactivation domain (ref. 37), respectively.  
*B*, GST-pulldown: Purified GST (lanes 1,2 & 7,8), GST-ARNO-PH-domain (GST-PHD; lanes 3,4 & 9,10) or GST-ARNO (lanes 5,6 & 11,12) (10 µg each) were incubated in duplicate with a purified fusion protein comprising MBP and the carboxyl terminus of the A<sub>2A</sub>-receptor (10 µg) in 0.2 mL buffer; proteins bound to GSH-sepharose were released by denaturation; aliquots (20% each) were loaded on two separate polyacrylamide gels, electrophoretically resolved and transferred to nitrocellulose membranes, which were probed either with an antiserum directed against MBP (lanes 1 to 6) or directed against GST (lanes 7-12). Note that GST-ARNO is subject to substantial degradation.

**Fig. 2. Confocal and FRET microscopy of A2A-adenosine receptors and ARNO/cytohesin-2.**  
*A*, HEK-293 were transfected with equal amounts of vectors coding for a yellow fluorescent protein tagged version of the A<sub>2A</sub>-receptor (YFP-A<sub>2A</sub>R) and a cyan fluorescent protein version of ARNO/cytohesin-2 (CFP-ARNO). The next day images were captured by laser-scanning microscopy. Shown are representative single planes captured for the YFP-tagged A<sub>2A</sub>-receptor (left) or CFP-tagged ARNO (middle); the overlay is shown on the right panel.  
*B*, Cells were transfected with plasmids encoding for CFP-ARNO plus YFP-A<sub>2A</sub>R. The next day images were taken using an acceptor filter set (CFP-filter) before (left) and after (middle) photobleaching. The ratio image (right) represents the division of the image after by the image before photobleaching. An YFP filter was used to visualize cells expressing A<sub>2A</sub>-receptors (left). Note that cells co-expressing CFP-ARNO and YFP-A<sub>2A</sub>R clearly show a positive FRET in the ratio image along their cell membrane (white arrows) but cells expressing only YFP- A<sub>2A</sub>R (white asterisk) do not show any FRET. The inserted bars correspond to 10 µm.

**Fig. 3. Inducible expression of wild type ARNO/cytohesin-2 and dominant negative E156K-ARNO/cytohesin-2 in HEK 293 cells that stably express the A<sub>2A</sub>-adenosine receptor.**  
*A*, Cellular lysates were prepared from HEK 293 cells stably expressing the A<sub>2A</sub>-receptor (lanes 4 & 5), or these cells subjected to transient transfection with a plasmid driving the expression of GFP-tagged ARNO/cytohesin-2 (lane 1), wild type ARNO/cytohesin-2 (lane 2) or dominant negative E156K-ARNO/cytohesin-2 (lane 3). The amount of protein loaded on to the SDS polyacrylamide gel was 20 µg (lanes 1 & 5) and 10 µg (lanes 2 - 4). After electrophoretic transfer to nitrocellulose membranes, immunodetection was done with an antiserum directed against ARNO/cytohesin-2.  
*B*, HEK 293 cells stably expressing the human A<sub>2A</sub>-receptor and, in addition, wild type ARNO/cytohesin-2 (wt-ARN-A<sub>2A</sub>) or dominant negative E156K-ARNO/cytohesin-2 (dn-ARN-A<sub>2A</sub>) under the control of GAL4DBD/hPRLBD/p65AD were treated for the indicated times with 10 nM mifepristone. Cellular lysates (10 µg) were probed for the expression of ARNO/cytohesin-2 by immunoblotting as in Panel A.  
*C*, Co-immunoprecipitation of the A<sub>2A</sub>-receptor and ARNO/cytohesin-2: Cells from a wt-ARN-A<sub>2A</sub> cell clone (lane 1 – 3) were induced to express
ARNO/cytohesin-2 by incubation with 10 nM mifepristone (lane 1 and 2) or were treated with vehicle (lane 3) for 8 h. HEK 293 cells expressing ARNO/cytohesin-2 under the control of GAL4DBD/hPRLBD/p65AD were treated with 10 nM mifepristone for 8 h (lane 4). Cells were lysed and membranes (0.2 mg) were extracted with detergent containing buffer and immunoprecipitation was done as outlined under "Experimental Procedures".

Fig. 4. Agonist-induced accumulation of [3H]cAMP after inducible expression of wild type ARNO/cytohesin-2 and dominant negative E156K-ARNO/cytohesin-2 in HEK 293 cells that stably express the A2A-adenosine receptor. A & B, Membranes (100 µg/assay) were prepared from HEK 293 cell clones stably expressing the human A2A-receptor and, in addition, wild type ARNO/cytohesin-2 (A) or dominant negative E156K-ARNO/cytohesin-2 (B) under the control of GAL4DBD/hPRLBD/p65AD and incubated with the indicated concentrations of the antagonist radioligand in 0.2 mL buffer containing 8 U/mL adenosine deaminase and 100 µM GTPγS; non-specific binding determined in the presence of 10 µM XAC was <10% of total binding at the highest radioligand concentration employed and was subtracted. Prior to homogenization, the cells had been incubated in the absence (closed symbols) and presence of 10 nM mifepristone (open symbols) for 8 hours to induce expression of wild type ARNO/cytiohesin-2 and dominant negative E1516K-ARNO/cytohesin-2. Data are from one representative experiment carried out in duplicate which was repeated three times with similar results. C & D, HEK 293 cell clones from Panels A and B were metabolically labeled with [3H]adenine for 16 h and – where indicated by the open symbols – concomitantly pretreated with mifepristone for 8 hours. After addition of fresh medium, the cells were stimulated with the indicated concentrations of CGS21680 for 30 min; subsequently cells were lysed and [3H]cAMP was resolved from ATP by sequential chromatography on Dowex AG 1X-8 and neutral alumina. E & F, The cell clones were induced to express ARNO/cytohesin-2 and metabolically labeled with [3H]adenine as in Panels C and D. After removal of the medium cells were pretreated for two hours with 1 µM CGS21680 to induce desensitization (open symbols); the agonist was subsequently removed by repetitive washes and the cells (open symbols) were then rechallenged with the indicated concentrations of CGS21680. Alternatively, control cells were stimulated with agonist without any prior desensitization (closed symbols). Each data point corresponds to means from three dishes; error bars indicate S.D. Two additional experiments gave similar results.

Fig. 5. Agonist-induced stimulation of MAP-kinase after inducible expression of wild type ARNO/cytohesin-2 and dominant negative E156K-ARNO/cytohesin-2 in HEK 293 cells that stably express the A2A-adenosine receptor. HEK 293 cell clones stably expressing the human A2A-receptor and, in addition, wild type ARNO/cytohesin-2 (A & B) or dominant negative E156K-ARNO/cytohesin-2 (C & D) under the control of GAL4DBD/hPRLBD/p65AD were rendered quiescent by serum starvation for 16 h and incubated in the absence (A & C) and presence of 10 nM mifepristone (B & D) for 8 hours. Subsequently, the agonist CGS21680 (1 µM) was added for the indicated time. Aliquots of cellular lysates (10 µg protein) were applied to SDS polyacrylamide gels. After electrophoretic resolution and transfer to nitrocellulose, the level of active MAP-kinase was assessed by immunoblotting with an antiserum recognizing the dually phosphorylated, active enzyme (upper panels, P-erk). In parallel, the total level of p42 and p44 MAP-kinase was determined as loading control (lower panels, holo-erk). Bar diagrams show the densitometric quantification of the immunoreactivity normalized to unstimulated cells from four independent experiments; error bars indicate S.D.

Fig. 6. Brefeldin A treatment of HEK 293 cells that stably express the A2A-adenosine receptor. HEK 293 cell clones stably expressing the human A2A-receptor were rendered
quiescent by serum starvation and incubated in the absence (A) and presence of 5 µg/ml brefeldin A (B) for 30 min. Subsequently, the agonist CGS21680 (1 µM) was added for the indicated time. Aliquots of cellular lysates (10 µg protein) were applied to SDS polyacrylamide gels. After electrophoretic resolution and transfer to nitrocellulose, the level of active MAP-kinase was assessed by immunoblotting with an antiserum recognizing the dually phosphorylated, active enzyme (upper panels, P-erk). In parallel, the total level of p42 and p44 MAP-kinase was determined as loading control (lower panels, holo-erk). Bar diagrams show the densitometric quantification of the immunoreactivity normalized to unstimulated cells from three independent experiments; error bar indicate S.D. C, Cells were transfected with a fluorescent Golgi-marker and treated with brefeldin A as described above. Pictures of paraformaldehyde fixed cells were taken before (a,b), after 30 min (c) and 90 min of addition of brefeldin A; the white bar corresponds to 10 µm.

Fig. 7. The effect of a dominant negative variant of Arf 6 on agonist-induced stimulation of MAP-kinase in HEK 293 cells that stably express the A2A-adenosine receptor. HEK 293 cell clones stably expressing the human A2A-receptor were subjected to transient transfection with a plasmid driving the expression of a dominant negative version of Arf 6 (T27N). Cells were rendered quiescent by serum starvation for 16 hours. Subsequently, the agonist CGS21680 (1 µM) was added for the indicated time. Aliquots of cellular lysates (10 µg protein) were applied to SDS polyacrylamide gels. After electrophoretic resolution and transfer to nitrocellulose, the level of active MAP-kinase was assessed by immunoblotting with an antiserum recognizing the dually phosphorylated, active enzyme (upper panels, P-erk). In parallel, the total level of p42 and p44 MAP-kinase was determined as loading control (lower panels, holo-erk). Bar diagrams show the densitometric quantification of the immunoreactivity normalized to unstimulated cells from three independent experiments; error bar indicate S.D.
Fig. 1

**A**

**B**

WB: anti-MBP

WB: anti-GST

- 75kD
- 50kD
- 37kD

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Fig. 2

A

YFP-A$_{2A}$R  CFP-ARNO  overlay

B

YFP-filter  CFP-filter before bleaching  ratio CFP after/before bleaching
Fig. 3

A

B

C

wtARNO
dnARNO

A2A-flag + + + + -
ARNO + + - + +
Co-IP - + + + +
Lysate + - - - -

IP: α-flag
WB: α-ARNO

50kD
37kD
Fig. 4

A

B

C

D

E

F

[³H]-cAMP (cpm)

[³H]-cAMP (cpm)

[³H]-cAMP bound (nmol/mg protein)

[³H]-cAMP bound (nmol/mg protein)

ARNO (wt)

ARNO (E156K)

CGS21680 (nM)

CGS21680 (nM)

ARNO (wt)

ARNO (E156K)

CGS21680 (nM)

CGS21680 (nM)

ARNO (wt)

ARNO (E156K)

CGS21680 (nM)

CGS21680 (nM)

ARNO (wt)

ARNO (E156K)

CGS21680 (nM)

CGS21680 (nM)
Fig. 5

A

wtARNO

α-P-ERK

α-holo-ERK

wtARNO

unind

B

wtARNO

induced

C

dnARNO

unind

D

dnARNO

induced
Fig. 6

A

α-P-ERK  
α-holo-ERK  

Control

B

α-P-ERK  
α-holo-ERK

+ BFA

C
Fig. 7

A

0 2 5 10 20 30 45 60 75 90 min

α-P-ERK
α-holo-ERK

Control

B

0 2 5 10 20 30 45 60 75 90 min

α-P-ERK
α-holo-ERK
dnArf6
Heterotrimeric G protein independent signaling of a G protein-coupled receptor: Direct binding of arno/cytohesin-2 to the carboxyl terminus of the A2A-adenosine receptor is necessary for sustained activation of the ERK/MAP-kinase pathway
Ingrid Gsandtner, Christoforos Charalambous, Eduard Stefan, Egon Ogris, Michael Freissmuth and Jürgen Zezula

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