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Adenosine and Gastrointestinal Inflammation

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Abstract

Nucelosides such as adenosine (Ado) influence nearly every aspect of physiology and pathophysiology. Extracellular nucleotides liberated at local sites of inflammation are metabolized through regulated phosphohydrolysis by a series of ecto-nucleotidases including ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5′-nucleotidase (CD73), found on the surface of a variety of cell types. Once generated, Ado is made available to bind and activate one of four G-protein-coupled Ado receptors. Recent *in vitro* and *in vivo* studies implicate Ado in a broad array of tissue protective mechanisms that provide new insight into adenosine actions. Studies in cultured cells and murine tissues have indicated that Ado receptors couple to novel post-translational protein modifications, including Cullin deneddylation, as a new anti-inflammatory mechanism. Studies in Ado receptor-null mice have been revealing and indicate a particularly important role for the Ado A2B receptor in animal models of intestinal inflammation. Here, we review contributions of Ado to cell and tissue stress responses, with a particular emphasis on the gastrointestinal mucosa.

Keywords

mucosa; inflammation; colitis; neutrophil; epithelium; endothelium; murine model

I. Introduction

Circulating or locally released nucleotides are rapidly metabolized by ecto-enzymes localized to the cell surface. Ecto-5′-nucleotidase (CD73) is a glycosyl phosphatidylinositol (GPI)-linked, membrane-bound glycoprotein which hydrolyzes extracellular nucleoside monophosphates into bioactive nucleoside intermediates[1]. Surface-bound CD73 metabolizes adenosine 5′-monophosphate (AMP) to adenosine, which when released can activate one of four types of G-protein coupled, seven transmembrane spanning adenosine receptors (AdoR) or can be internalized through dipyridamole-sensitive carriers[2]. Adenosine receptors are expressed on a wide variety of cells, and many cell types have been shown to express more than one isoform of the receptor. Likewise, activation of surface AdoR has been shown to regulate diverse physiologic endpoints. In the recent years, our understanding of nucleotide metabolic pathways has benefited from the development of genetically manipulated animals, particularly mice genetically deficient in the various adenosine receptors. Here, we review relevant studies addressing physiologic influences of Ado within the gastrointestinal mucosa utilizing a variety of model systems.

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Disclosure

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II. Functional Mucosal Responses Mediated by Adenosine

The gastrointestinal tract is lined by functionally differentiated epithelial cells. Primary physiologic functions of the epithelium include barrier function, absorption and mucosal hydration. Mucosal hydration is accomplished through a coordinated series of ion transport events. As part of a tissue adaptive response, a number of purine nucleotide metabolites, including adenosine, have been implicated to influence epithelial electrogenic chloride secretion, the transport event responsible for mucosal hydration. This aspect of epithelial function has been studied in detail utilizing models of intact epithelial cell layers coupled with electrophysiologic strategies. Studies by Madara et al. examining biological properties of soluble mediators derived from activated inflammatory cells (e.g. neutrophils and eosinophils) identified a small, protease-resistant fraction originally termed neutrophil-derived secretagogue (NDS)[3, 4]. When incubated with epithelia, NDS potently activated electrogenic chloride secretion and associated fluid transport in cultured intestinal epithelial cells. Subsequent biochemical analysis of NDS identified this molecule to be 5-AMP[4]. With no known AMP receptor, studies turned toward defining potential 5′-AMP metabolic pathways. These studies revealed the expression of CD73 as a critical control point for the metabolism of 5′-AMP to Ado[5]. CD73 was shown to be expressed at a very high level on the apical aspect on intestinal epithelial cells. The only known function of CD73 is the phosphohydrolysis of AMP to Ado. From such observations, biochemical and pharmacologic studies demonstrated the expression of the Ado A2B receptor (A2BAR) isoform of adenosine receptors on cultured and primary intestinal epithelial cells and in human tissue as the primary determinant for mucosal hydration[6]. A mechanism of such fluid transport mediated by Ado is shown in Figure 1.

A number of studies have also implicated adenosine receptors in the control of tissue barrier function. For example, A2BAR, which couples via G proteins Gs and possibly Gq[7, 8], has been demonstrated to be critical for the restitution of epithelial and endothelial monolayers following transmigration. Successful transmigration of leukocytes across endothelial and epithelial cells is accomplished by temporary PMN self-deformation with localized widening of the inter-junctional spaces[9, 10], a process with the potential to disturb endothelial and epithelial barrier function. Original studies by Lennon et al. revealed that the prominent signaling pathway for closing inter-endothelial gaps during transmigration involved adenosine-stimulated “resealing” of the barrier[11]. Subsequently, it was shown that protein kinase A signaling through the A2BAR recruits the vasodilator-stimulated phosphoprotein (VASP), a protein originally identified in platelets implicated in actin-binding and cross-linking functions[12]. This work demonstrated that VASP is phosphorylated via a PKA-dependent process in conditions that enhance barrier recovery following epithelial [13] and endothelial [14] disruption. Confocal microscopy studies revealed that VASP localizes with ZO-1 at epithelial tight junctions and at cell-cell borders and that phosphorylated VASP appears at the junction during epithelial and endothelial restitution. Subsequent transfection studies utilizing epithelial cells expressing truncated forms of VASP abnormal in oligomerization or actin-binding activity revealed a functional diminution of barrier recovery [13, 14] (see Figure 2).

III. Mucosal Sources of Adenosine

In ongoing inflammation, a number of cell types actively release adenine nucleotides, primarily in the form of ADP and ATP [15-17]. Inflammatory cells can be a rich source of nucleotides, and given the association of neutrophils with adenine-nucleotide/nucleoside signaling in the inflammatory milieu[4, 11], it was hypothesized that PMNs could represent a potential source of extracellular ATP [18, 19]. Initial studies utilizing luminometric ATP detection assays from supernatant fractions revealed that PMNs release ATP in an

activation-dependent manner. Pharmacological strategies identified a mechanism involving connexin 43 (Cx43) hemichannels as the membrane pore for ATP release. Cx43 molecules assemble as hexameric "connexons" that form junctional complexes between different cell types[20] and various leukocytes have been shown to express Cx43[21]. More recent studies implicated Cx43 connexons as intercellular signaling channels via the release of ATP [20, 22]. PMNs from induced *Cx43*$^{-/-}$ mice, whereby activated PMNs release less than 15% of ATP relative to littermate controls and Cx43 heterozygote PMNs were intermediate in their capacity for ATP release. This study implicated Cx43 in activated PMN ATP release, therein contributing to the innate metabolic control of the inflammatory milieu [18]. Subsequent studies by others revealed that human neutrophils release ATP predominantly from the leading edge of their cell surface as a mechanism to amplify chemotactic signals and direct cell orientation by feedback through P2Y2 nucleotide receptors [23, 24].

Activated platelets are also known to release nucleotides at high concentration [25]. In this context, Weissmuller et. al. highlighted the interaction between PMN and platelets in regulating intestinal inflammation and fluid transport via nucleotide release [26]. Mucosal diseases are often characterized by a mixed inflammatory infiltrate that includes PMNs and platelets. These studies showed that platelets migrate across intestinal epithelial cells in a PMN-dependent manner. Furthermore, platelet-PMN co-migration was observed in intestinal tissue derived from human patients with inflammatory bowel disease (IBD). The translocated platelets were found to release large quantities of ATP, which was metabolized to adenosine via a 2-step enzymatic reaction involving CD73 and CD39-like molecules expressed on intestinal epithelial cells (IEC). Subsequent studies revealed a mechanism involving Ado-mediated activation of electrogenic chloride secretion, with concomitant water movement into the intestinal lumen[4]. Together, these studies demonstrated that ecto-NTDases are expressed on IEC and interact with platelet-derived nucleotides through a mechanism involving platelets that "piggy back" across mucosal barriers while attached to the surface of PMN [26] (Figure 1).

IV. Mechanisms of Adenosine Action

It is long appreciated that G protein-coupled receptors, particularly those which elevate intracellular cAMP (i.e A2AAR and A2BAR), can serve as sensors of inflammation and promote tissue protective responses[27]. Less is known about post-receptor events and molecular mechanisms of action [28]. It is recently appreciated that Ado receptor signaling is linked to a post-translational modification termed neddylation, the reversible conjugation of the ubiquitin-like NEDD8 (Neural precursor cell expressed, developmentally down-regulated 8) [29] moiety to proteins [30]. Neddylation and deneddylation responses are highly conserved and exist in a wide variety of cell types [31] and species [32-35]. Activation of the Nedd8-precursor through cleavage a carboxy-terminal glycine residue by UCH-L3 (also called SENP8) enables conjugation to the E1 UBA3-APPBP1 heterodimer, also known as Nedd8-Activating-Enzyme (NAE) [36-39]. Subsequently NEDD8 is conjugated to its specific E2 Ubc12 (ubiquitin conjugating enzyme) [40] and afterwards linked to the E3 complex [41, 42] (Figure 3). Neddylation plays an essential role in the post-translational modification of Cullin-RING-ligases [43] involved in the ubiquitin pathway. Cullins function as scaffolding proteins and are essential for the assembly of the ubiquitin E3 ligase complex conjugating ubiquitin to target proteins and thus marking them for proteasomal degradation, including IκB, which leads to translocation of the NFκB p50/p65 heterodimer to the nucleus [44, 45]. Of note, the ubiquitin E3 ligase complex integrating Cullin-2 is central to the regulation of hypoxia-inducible factor[31], a molecule which has gained significant attention as a protective factor in various models of mucosal inflammation[46].

Ado potently inhibits the neddylation of Cullin-1 in endothelial and epithelial cells [47]. Insights into potential roles for Cullin-deneddylation in inflammation were provided by Neish et al. who demonstrated that commensal bacteria-associated attenuation of NFκB is Cullin-de-neddylation-dependent in epithelial cells [48]. Furthermore, Kumar et al. demonstrated that commensal bacteria influence the neddylation status of Cullin-1 (Cul-1) through generation of reactive oxygen species (ROS)[49]. Initially it was demonstrated that epithelial cells elicit increases in ROS when co-cultured with commensal bacteria. This resulted in a transient and reversible deneddylation of Cul-1 and subsequent decrease of NFκB pathway end products. Interestingly, they were able to show that different commensal bacterial strains differ in the amount of ROS they generate. Since an altered microbiota is a prevalent observation in patients with IBD[50] differing amounts of ROS in individual locations within the intestine may altering the inflammatory response in IBD [51].

One particularly intriguing mechanism suggests that Ado inhibits NF-κB through actions on proteasomal degradation of IκB proteins [47]. These findings were based on studies addressing adenosine signaling mechanisms which revealed that adenosine and adenosine analogs display a dose-dependent deneddylation of Cul-1 with rank order of specificity A2BAR >A1AR>>A2AAR = A3AR [47]. Current understanding indicates that deneddylation reactions on Cullin targets via CSN-associated proteolysis is increasingly implicated as a central point for Cullin-mediated E3 ubiquitylation [43]. Notably, other pathways for deneddylation have been reported. For example, signaling elicited by adrenomedullin, which is induced in LPS-associated inflammation[52], deneddylated Cul-1 with beneficial influences in a murine model of IBD [53]. Furthermore, the identification of the Nedd8-specific protease SENP8 has provided new insight into this emerging field. SENP8 appears to contain isopeptidase activity capable of directly deneddylating Cullin targets [54, 55]. Moreover, recent studies indicate that SENP8 is central for inflammatory responses at the vascular interface [56]. Pharmacological targeting of the neddylation pathway, more specifically the NAE, using MLN4924[57], a structural homolog of 5′-AMP, significantly abrogated NF-κB responses and reduced secretion of LPS-elicited and TNF-α-elicited pro-inflammatory cytokines in human endothelial cells[56] as well as in human and murine macrophage cells lines [58]. Likewise, MLN4924 abrogated pro-inflammatory responses to LPS *in vivo* [56]. These studies identify SENP8 as a proximal regulator of Cullin neddylation and provide an important role for SENP8 in fine-tuning of the inflammatory response (see Figure 3).

IV. The role of individual Ado receptors (ARs) in GI inflammation *in vivo*

The rather broad expression pattern of individual AdoR in the GI tract have engendered significant interest as therapeutic targets in GI inflammation. A number of studies have addressed the contribution of individual ARs (A1AR, A2AAR, A2BAR, or A3AR) to gastrointestinal inflammation in murine models. Specifically, the availability of AdoR-null mice and selective agonists and antagonists have revealed important insight into the potential contributions of Ado to GI inflammation.

While less is known about A1AR in GI disease models, the A2A and A2BAR receptors have been studied in some detail [59-61]. The A2AAR is critical for A2AAR signaling in T cell-mediated regulation of colitis. Moreover, treatment with a specific A2AAR agonist attenuated the production of pro-inflammatory cytokines and attenuation of colitis [62]. In addition, recent studies have performed a “head-on” comparisons between *A2AAR*$^{-/-}$ and *A2BAR*$^{-/-}$ in murine colitis models [63]. Previous studies had revealed that neither *A2AAR*$^{-/-}$ nor *A2BAR*$^{-/-}$ animals manifest outward immunological defects when housed in specific pathogen-free conditions[64]. Herein, *A2AAR*$^{-/-}$ or *A2BAR*$^{-/-}$ mice were subjected to DSS protocols and compared their responses to littermate wild-type controls. Initial

studies using 3.5% DSS showed that *A2BAR*$^{-/-}$ mice became profoundly ill within 3 days of induction, associated with high mortality which required lowering the DSS concentration to 2.5%. *A2AAR*$^{-/-}$ animals remained healthy at 2.5% DSS with no significant differences observed in weight loss curves between *A2AAR*$^{-/-}$ mice and littermate controls. Notably, *A2AAR*$^{-/-}$ mice showed increased susceptibility to higher concentrations of DSS. Indeed, when DSS concentrations were increased from 2.5% to 4.5%, *A2AAR*$^{-/-}$ showed significantly increased weight loss on days 2 – 6 and increased colon contraction compared to wild-type controls. Thus, it would appear that both *A2AAR*$^{-/-}$ and *A2BAR*$^{-/-}$ mice have increased susceptibility to DSS colitis, but that this phenotype is likely more severe in *A2AAR*$^{-/-}$[63]. Likewise, enteral administration of the selective A2BAR inhibitor PSB1115 resulted in a similar increase in severity of DSS colitis.[63] Together, these studies indicate a central regulatory role for the A2BAR in modulating the acute inflammatory phase of DSS colitis [63]. These studies are consistent with other studies that found a more severe phenotype of *A2BAR*$^{-/-}$ mice during intestinal ischemia-reperfusion injury[65] and implicate the A2BAR agonist BAY 60-6583 in the treatment of this condition[65]. Moreover, other studies from our laboratory have shown attenuation of mucosal inflammation during acute lung injury[61] or during intestinal hypoxia exposure[60, 64] mediated by the A2BAR. Similarly, A2BAR signaling is protective during renal[66] or myocardial ischemia[67, 68]. This is also consistent with several studies from other investigators, indicating A2BAR signaling in protection from vascular inflammation[69, 70], inflammation during organ transplantation[71] or attenuation of pulmonary inflammation during hypoxia[47].

By contrast, some studies have suggested a pathological role of A2BAR signaling in murine colitis [72, 73] and in *Clostridium difficile* intoxication[74]. Exposure of mice to DSS induced colitis have revealed that *A2BAR*$^{-/-}$ are protected and mucosal inflammation is attenuated. Such observations would be consistent with previous studies in cultured epithelial indicating more a pathological role for A2BAR signaling, including the observations that TNF strongly induces A2BAR expression [75]and the findings that A2BAR signaling enhances IL-6 and other pro-inflammatory cytokines [76-78].

Why the results from this study[73] might be different from our own [63, 65] is not readily apparent. In contrast to the study by Kolachala et al.,[73] several previous studies support an anti-inflammatory and tissue protective role of A2BAR signaling in different organ systems. As outlined above, gene-targeted mice for the A2BAR show enhanced vascular inflammation when exposed to endotoxin[70] or during acute vascular injury.[69] Similarly, gene-targeted deletion of the A2BAR is associated with enhanced vascular leak and inflammation during hypoxia.[60, 64, 79] Potential explanations why the studies of Kolachala et al.[72, 73] found a detrimental role of A2BAR signaling during murine colitis could include details in the colitis protocol, differences in murine strains with genetic deletion of the A2BAR or differences of the animal facilities resulting in microbial flora that influence disease outcome. In this regard, it is well documented differences in the microflora can fundamentally change the course of disease in various mouse models of colitis[80]. It is important to note that in some of their studies, *A2BAR*$^{-/-}$ mice showed a strongly a pro-inflammatory phenotype[73]. For example, *A2BAR*$^{-/-}$ mice showed increased susceptibility to systemic *Salmonella* infection, where >90% of *A2BAR*$^{-/-}$ mice died within 10 days compared with 20% of wild-type mice following orally administered *S typhimurium*. Consistent with the mortality data, *A2BAR*$^{-/-}$ mice also showed signs of weight loss earlier than WT mice[73]. In this regard, it is also possible that the lack of A2BAR signaling in *A2BAR*$^{-/-}$ could fundamentally influence innate immune cell function. For example, it has been shown that A2BAR contributes significantly to the differentiation of dendritic cells (DC)[81]. Ado-differentiated DC resulted in a pro-angiogenic, pro-inflamamtory and impaired allostimulatory phenotype. Thus, at the present time, it remains unclear exactly how the A2BAR functions in complex mucosal inflammatory models. Likely, additional

comparisons between the individual mouse strains may be necessary to rectify some of these discrepancies.

Finally, studies in $A3AR^{-/-}$ mice have proven quite interesting and potentially revealed a specific role for A3AR in the resolution of GI inflammation. For example, Butler et al. and Ren et al. have recently shown that $AA3R^{-/-}$ show overall decreased pathology in acute DSS colitis[82], but these animals failed to resolve inflammation-associated with chronic inflammation in this model[82]. Likewise, the adenosine A3 receptor agonist, N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide, has proven to be protective in at least two murine models of colitis.[83] In humans, A3AR expression negatively correlates with acute inflammatory score, Crohn's Disease Activity Index (CDAI) and disease chronicity[84], strongly implicating a need for more studies with this receptor subtype.

Conclusions

The gastrointestinal mucosa represents a unique environment for which to study nucleotide metabolism. The dynamic nature of multiple cell types in a highly proliferative area provides unique opportunities to define novel molecular mechanisms. Studies in animal models of IBD have revealed key seminal observations to the contribution of Ado receptors in mucosal immunology. More recent work has identified new molecular mechanisms of Ado receptor activation, particularly those related to post-translational modificaton of key effector molecules. Such findings provide a promising template for both drug discovery as well as novel endogenous adaptive pathways that reveal windows into the relevance of metabolism to disease processes.

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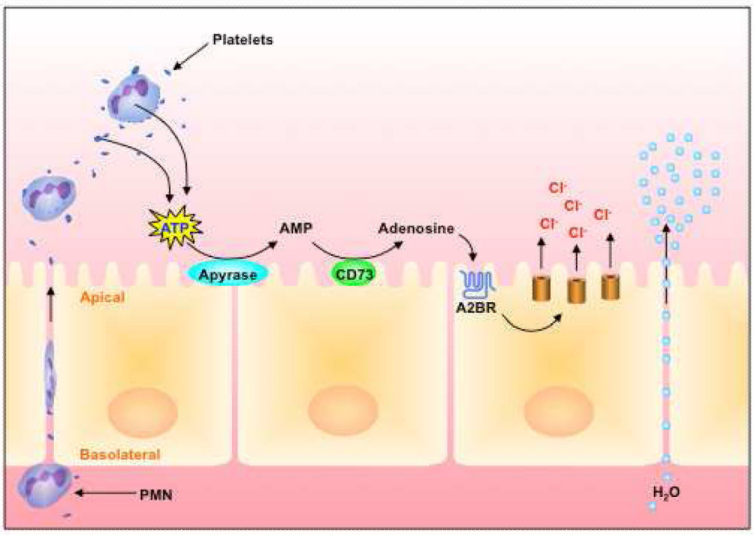

Figure 1. Coordinated activation of mucosal hydration during inflammation

During active inflammation, PMN transmigration (in conjunction with platelets, see text) migrate across the apical side of intestinal epithelial cells. PMN and platelet-derived ATP is metabolized to adenosine by a two-step enzymatic reaction involving ecto-apyrase (CD39 and CD39-like enzymes) and ecto-nucleotidase (CD73). Adenosine binding to epithelial adenosine A2B receptors results in activation of electrogenic Cl secretion and the paracellular movement of water. Such platelet/PMN - epithelial crosstalk pathway may serve as a defensive response by which mucosal surfaces are flushed from bacteria and bacterial products under inflammatory conditions.

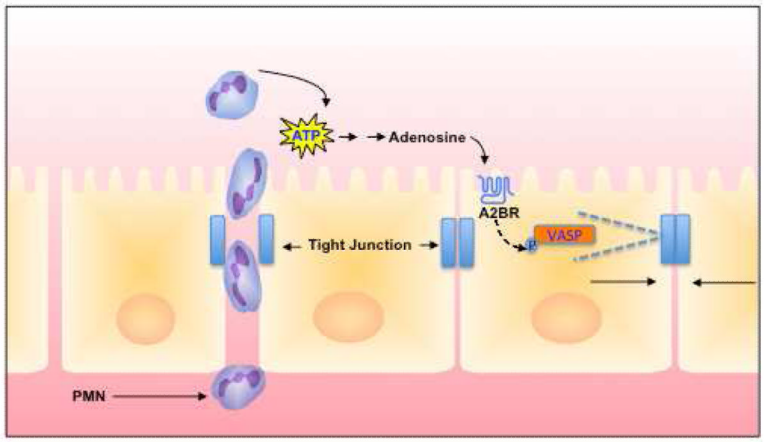

Figure 2. Model of adenosine-mediated epithelial restitution following PMN migration

Adenosine-mediated signaling increases levels of cAMP in epithelial cells and results in the activation of protein kinase A (PKA), and subsequent phosphorylation of VASP. In this model, phospho-VASP interactions with actin and tight junction proteins contribute to diminishing paracellular permeability through relaxation of actin cytoskeletal tension. Such a mechanism contributes to the resealing of epithelial monolayers following PMN transmigration.

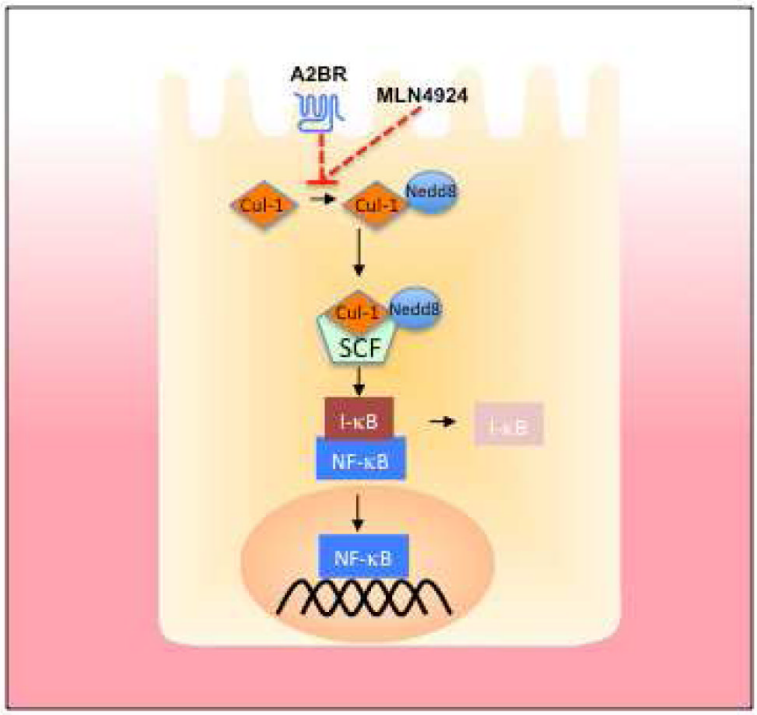

Figure 3. Molecular mechanism of anti-inflammation by adenosine

The canonical NFκB pathway is activated through the phosphorylation of IκB. This phosphorylation allows for its recognition by the neddylated Skp-Cullin-F-Box (SCF) complex, polyubiquitination and subsequent proteasomal degradation. Neddylation of Cul-1 is achieved through a multienzyme process, conjugating a NEDD8 moiety to the target protein (see text). Adenosine receptor activation, as well as the neddylation inhibitor MLN4924, inhibit Cullin neddylation or promote deneddylation of Cullin-proteins, thereby inhibiting the overall function of the SCF complex.