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p62 (SQSTM1) and cyclic AMP phosphodiesterase-4A4 (PDE4A4) locate to a novel, reversible protein aggregate with links to autophagy and proteasome degradation pathways

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ABSTRACT

Chronic challenge of cyclic AMP phosphodiesterase-4A4 (PDE4A4) with certain PDE4 selective inhibitors causes it to reversibly form intracellular aggregates that are not membrane-encapsulated. These aggregates are neither stress granules (SGs) nor processing bodies (PBs) as they contain neither PABP-1 nor Dcp1a, respectively. However, the PDE4 inhibitor rolipram decreases arsenite-induced SGs and increases the amount of PBs, while arsenite challenge ablates rolipram-induced PDE4A4 aggregates. PDE4A4 aggregates are neither autophagic vesicles (autophagosomes) nor aggresomes, although microtubule disruptors ablate PDE4A4 aggregate formation. PDE4A4 constitutively co-immunoprecipitates with p62 protein (sequestosome1, SQSTM1), which locates to both PDE4A4 aggregates and autophagosomes in cells constitutively challenged with rolipram. The mTor inhibitor, rapamycin, activates autophagy, prevents PDE4A4 from forming intracellular aggregates and triggers the loss of bound p62 from PDE4A4. siRNA-mediated knockdown of p62 attenuates PDE4A4 aggregate formation. The p62-binding protein, light chain 3 (LC3), is not found in PDE4A4 aggregates. Blockade of proteasome activity and activation of autophagy with MG132 both increases the level of ubiquitinated proteins found associated with PDE4A4 and inhibits PDE4A4 aggregate formation. Activation of autophagy with either thapsigargin or ionomycin inhibits PDE4A4 aggregate formation. Inhibition of autophagy with either wortmannin or LY294002 activates PDE4A4 aggregate formation. The protein kinase C inhibitors, RO 320432 and GO 6983, and the ERK inhibitors UO 126 and PD 98059 all activated PDE4A4 aggregate formation, whilst roscovitine, thalidomide and the tyrosine kinase inhibitors, genistein and AG17, all inhibited this process. We suggest that the fate of p62-containing protein aggregates need not necessarily be terminal, through delivery to autophagic vesicles and aggresomes. Instead, we propose a novel regulatory mechanism where a sub-population of p62-containing protein aggregates would form in a rapid, reversible manner so as to sequester specific cargo away from their normal, functionally important site(s) within the cell. Thus an appropriate conformational change in the target protein would confer reversible recruitment into a sub-population of p62-containing protein aggregates and so provide a regulatory function by removing these cargo proteins from their functionally important site(s) in a cell.

1. Introduction

Cyclic AMP is a universal second messenger that controls many key physiological processes [1]. It is now well-appreciated that cAMP signalling is compartmentalised in cells [2–4]. Gradients and pools of intracellular cAMP are sculpted by sequestered cAMP phosphodiesterase isoforms acting on cAMP generated by adenylyl cyclase isoforms restricted to sub-domains of the cell plasma membrane [2,5–8]. A range of PKA and EPAC sub-populations anchored at specific intracellular sites then interpret gradients of cAMP and transduce them to locally sequestered target molecules [1,9]. Much of our appreciation of the critical role of targeted cAMP degradation in compartmentalised cAMP signalling has come from the discovery that diversity among the cAMP phosphodiesterase-4 (PDE4) isoforms provides a toolbox for creating species that can be directed to specific intracellular locales, invariably through sequestration to signalling scaffold proteins such as tyrosyl kinases, RACK1, myomegalin, β-arrestins, AKAPs, DISC1 and Ndel [2,5,6,10]. This allows specific PDE4 isoforms to have distinct functional roles in cells, as uncovered by the use of dominant negative strategies [11–15]. Indeed, the importance of members of the four gene PDE4 family and the 25+ isoforms encoded is that selective inhibitors are

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being developed as therapeutics, acting as potent anti-inflammatory agents, anti-depressants, cognitive enhancers and anti-cancer agents [16–19]. However, therapeutic deployment of PDE4 selective inhibitors has been hampered due to efficacy issues, in part reflecting side effects such as nausea, emesis and a type of vasculitis [17–19], but also the realisation that at least certain PDE4 isoforms can adopt distinct conformational states that show very different affinities for interaction with certain types of PDE4 inhibitors [5,16,20–25]. Such distinct conformational states of PDE4 enzymes can be engendered in a number of ways that, to date, include their interaction with specific partner proteins [24,26,27], phosphorylation [22,25], ubiquitination [28], sumoylation [29], dimerisation [20,30] and the nature of the isoform-specific N-terminal region [31–35]. The functional consequences for the range of conformational changes elicited include changes in activity/activation, association with partner proteins, stability and inhibitor sensitivity. In the latter regard, the fact that PDE4 from various tissues can adopt markedly different sensitivities to the archetypal PDE4 selective inhibitor, rolipram, has attracted considerable interest in view of the possibility that this may relate to unwanted Side-effects [5,16,23]. Unfortunately aggregation issues have conspired against obtaining structures of full-length PDE4 isoforms that might usefully address this. Until very recently, all of the structural studies on PDE4 have utilised the engineered, isolated catalytic unit [36,37]. This identifies extremely high conservation within the active site pocket of members of all four PDE4 sub-families, indicating inherent difficulties in generating sub-family specific inhibitors of any practical selectivity. However, uniquely, members of the PDE4 family contain highly conserved regulatory modules called UCR1 and UCR2 [38], which can both interact with each other and with the catalytic unit [39,40]. Recently, the structure of part of the regulatory UCR2 region docked onto the PDE4 catalytic site has been solved [20], which has led to the discovery that certain PDE4 selective inhibitors can elicit conformational changes in PDE4 by, it is likely [21], stabilising a dimeric state where part of the UCR2 from one monomer docks across ('caps') the active site of the other monomer. These studies provide the first structural insight that some, but not all, PDE4 selective inhibitors can stabilise distinct conformational states of PDE4 isoforms [20,21].

Of the large PDE4 enzyme family, the PDE4A4 isoform is of particular interest as it is up-regulated [41] in chronic obstructive pulmonary disease (COPD), a condition to which PDE4 inhibitor therapeutics are directed and which are proving potentially efficacious [16–18]. Furthermore, PDE4A4 [38,42] and its rodent ortholog, PDE4A5 [43], interact with the p75 neurotrophin receptor (p75NTR) [44] to inhibit fibrin breakdown, thereby potentiating scarring, which is a feature of COPD pathology [45]. Thus it is likely to be important either to inhibit PDE4A4 or to displace it from p75NTR. This will allow cAMP levels local to p75NTR to be elevated, thereby facilitating fibrin breakdown, which would be a useful property for the effective treatment of COPD. We have shown that chronic, but not short-term, challenge with rolipram and certain other, but not all, PDE4 selective inhibitors causes the specific recruitment of PDE4A4, but not other PDE4A isoforms, into cytosolic aggregates/foci within cells [34]. This process shows discrimination between the two enantiomeric forms of rolipram, with (*R*)-(−)-rolipram preferred over (*S*)-(+)-rolipram. It also involves initiation of a distinct conformational change that arises from the binding of an appropriate competitive inhibitor within the cAMP binding pocket. The effect of this so-called 'inside-out' signalling [34] can be 'tracked' by mutational analysis from residues in the binding pocket through to those on the surface of the catalytic unit. However, in addition to a surface on the catalytic unit it also depends on the unique N-terminal region of PDE4A4/5. Intriguingly, this aggregation process is cAMP independent, intimately depends on protein synthesis and is reversible. Nevertheless, the mechanisms underlying this dramatic and selective redistribution of PDE4A4 and its relevance to the biological action of those PDE4 selective inhibitors able to elicit this transformation are unknown. Potentially compounds able to elicit such reversible intracellular redistribution of PDE4A4 may possess 'bonus' activity by virtue of their capacity to remove the enzyme from functionally relevant intracellular compartments in addition to exerting competitive inhibitory action. Such compound-driven selective sequestration would thus be expected to elicit comparable functional effects in allowing cAMP levels to rise in spatially discrete compartments controlled by tethered PDE4A4 in such a manner as those seen in so-called dominant negative studies achieved by displacement of selective PDE4 isoforms through overexpression of cognate, catalytically inactive species [11–15]. Of course it is also possible that the ability of certain PDE4 selective inhibitors to trigger PDE4A4 aggregate formation may also underpin unwanted side effects of such a sub-set of PDE4 selective inhibitors.

There is great interest in the formation of cytoplasmic inclusion bodies, which can, seemingly, be generated in a number of critical conditions and are not just due to irreversible aggregates formed from mis-folded proteins. For example, while Type 2 stress (X-rays, genotoxic drugs) induces apoptosis through the stress-activated p38 and JNK MAPK (SAPK) pathways, Type 1 physiological stress (hypoxia, heat-shock and arsenite) initiates a phylogenetically conserved protection mechanism where stalled initiation complexes are dynamically routed by TIA-1 and TIAR into discrete cytoplasmic foci called stress granules (SGs) [46,47]. Such TIA proteins contain a glutamine-rich prion-related domain (PRD) that has been proposed to allow self-aggregation and thereby drive the assembly of SGs, through which such proteins can rapidly associate and disassociate [48]. SGs can thus be rapidly both assembled and disassembled and contain not only various eukaryotic initiation factors (eIFs) together with RNA-binding proteins such as TIA-1, G3BP and FMRP, but also a number of proteins that mediate splicing, transcription, adhesion, signalling and development. Indeed, overexpression of DISC1, a protein whose gene is linked to schizophrenia and which has been shown to interact with PDE4A4, induces the assembly of eIF3- and TIA-1-positive SGs [49]. Also, under conditions when chaperones fail to aid in protein refolding, the aggregated mis-folded proteins are invariably subject to degradation through the ubiquitin proteasome pathway. However, they can also be targeted into specialized "holding stations" called aggresomes [50]. Such aggresome formation is thought to provide a physiologic mechanism to regulate the levels of certain cellular proteins such as the signalling protein, inducible nitric oxide synthase (iNOS) [51]. Critical to the recruitment of such physiologic species to aggresomes is CHIP (carboxy terminus of Hsp70-interacting protein), which has a tetratricopeptide repeat (TPR) domain at its amino terminus and a U-box domain at its carboxy terminus. The ubiquitin ligase function of this protein is required in targeting pre-aggresomal structures to the aggresome through interaction with histone deacetylase 6 (HDAC6), which serves as an adaptor between ubiquitinated proteins and the dynein motor [52]. Such cytosolic aggregates can be subject to degradation by autophagy, offering a route for clearance of these species in which HDAC6 and microtubules have been implicated [53]. Such autophagic vesicles appear to be coated [54] with the autophagic marker light chain 3 (LC3) that binds directly to p62 protein (sequestosome 1 protein; SQSTM1; A170; ZIP) [55–57]. Indeed, p62 is thought to perform a shuttling role, recruiting proteins to aggresomes. This scaffold protein, namely p62, can polymerize via its N-terminal Phox and Bem1p (PB1) domains, bind aPKC through its PB1 domain, has a ZZ finger, binds Traf6 and binds K63-ubiquitinated species through its C-terminal UBA domain [58]. Thus p62 is detected in many ubiquitinated protein aggregates associated with important disease states such as the neurofibrillary tangles seen in Alzheimer disease, Lewy bodies in Parkinson disease and aggregates found in Huntington disease, for example [58,59]. Autophagy not only provides a route through which cytosolic, non-ubiquitinated forms of mis-folded and