Summary

Cyclic adenosine 3', 5'- monophosphate (cAMP) is a second messenger that functions through binding to its downstream targets protein kinase A (PKA), cyclic-regulated ion channels (CNG channels) and Epac (exchange protein directly activated by cAMP). Epac (Epac1 and Epac2) is a guanine nucleotide exchange factor towards both Rap1 and Rap2. It is kept in its inactive conformation by an intramolecular interaction between the regulatory and catalytic domain. Binding of cAMP to the cAMP-binding domain within the regulatory region liberates the catalytic domain, resulting in the activation of Epac. In order to visualize the conformational change between the inactive- and active-state of Epac, a CFP-Epac-YFP probe was generated and fluorescence resonance energy transfer (FRET) between the two fluorescent moieties was monitored in vivo. The FRET signal rapidly decreased in response to cAMP-raising agents, whereas it fully recovers after addition of cAMP-lowering agonists (Chapter 2). This indicates that cAMP causes a significant conformational change in vivo and supports the unfolding model for Epac1 activation. In addition, compared with the PKA-FRET probe, the Epac-FRET probe has a much larger dynamic range for cAMP, and this allows the Epac-FRET probe to measure changes in physiological cAMP levels which PKA-FRET probe failed to record (Chapter 2).

8-pCPT-2'-O-Me-cAMP (007), a cAMP analogue which can specifically activate Epac1 but not PKA has put Epac1 in a pivotal position in many biological processes such as VE-/ E-cadherin-mediated junction formation, integrin-mediated cell adhesion, insulin secretion and sodium proton exchange activity. However, the lack of suitable antibodies against Epac1 limited a detailed analysis of Epac. Therefore, a series of Epac1 antibodies (Abs) were generated and characterized. 5D3, one of the Epac1 monoclonal Abs, was further characterized. Both in vivo (data not shown) and in vitro experiments demonstrated that 5D3 can specifically recognize the active conformation of Epac1, and the epitope of 5D3 was mapped within the cAMP binding domain, in particular around Leucine 273. This region is hidden during autoinhibition (Chapter 3 and its Addenum).

To determine the subcellular localization of Epac1 was the main goal of my project. Using Epac1 Abs, we observed that Epac1 is mainly distributed around the perinuclear region, especially in the endoplasmatic reticulum and the Golgi apparatus, as well as in the plasma membrane, especially at microvilli in fully polarized cells (Chapter 4). Functional domains which are responsible for the proper localization of Epac1 were also analyzed in detail. Our results revealed that both the DEP domain and the EzB domain (the first 49 aa) are required for the correct localization of Epac1 and the activation of Rap. The EzB domain is not only responsible but also sufficient for targeting Epac1 at microvilli. In contrast the DEP domain is only responsible for membrane localisation (Chapter 4). Importantly, the microvillar localization is through binding to Ezrin/Radixin, proteins that function as linkers between the actin cytoskeleton and the apical membrane of polarized cells and as scaffold protein for protein complexes. The functional relevance of this interaction is still unclear, but the mere fact that Epac1 only binds to the active form of Ezrin/Radixin indicates that activation of these proteins is a crucial step in the spatial regulation of Epac1.

An interesting and surprising observation was that Epac1 accumulates in the nucleus when the EzB domain of Epac1 was deleted. This effect could be mimicked by stimulation of the cells with HGF, which induces cell scattering, and with overexpression of RapV12 which induces cell spreading. These results suggest that upon loss of cell polarity and thus disruption of the apical structures, part of Epac1 translocate to the nucleus. However, the function of Epac1 in the nucleus remains unclear (Addendum of Chapter 4).

结　论

Cyclic adenosine 3', 5'- monophosphate (cAMP)　是细胞内的一个非常重要的第二信使分子，它可以通过其下游的作用原件比如：PKA，cyclic-regulated ion channels (CNG channels)和 Epac (exchange protein directly activated by cAMP)　来发挥其功能。Epac 是小 G 蛋白 Rap 的激活因子之一。它包括一个调控区和催化区，并可以通过调控区和催化区之间的分子内结合（autoinhibition）而使 Epac 蛋白呈现非激活态（闭合状态）。当 cAMP 结合在其调控区的 cAMP 结合域时，可以破坏其自体抑制作用，从而使其催化区暴露出来，利于 Rap 小蛋白的结合，此时，Epac 则呈现了激活态（开放状态）。为了证明 Epac 蛋白的激活模型，我们构建了 Epac 的 FRET 载体，即将 YFP 和 CFP 银光蛋白融合在其氮端和碳端，检测 Epac 在从非激活态转变到激活态时，是否会降低从 CFP 到 YFP 的能量转移。试验结果表明，Epac 在非激活态时，氮端和碳端之间的距离非常接近以致于使 CFP 的辐射能量转移到邻近的 YFP，从而可以检测到 YFP 的辐射光谱。当 Epac 被激活时，氮端和碳端之间的距离由近变远，从而不能检测到由 CFP 辐射而转移到 YFP 的辐射光谱。因此，利用 Epac 的 FRET 载体，我们证实了 Epac 的激活模型就是一个由闭合到开放的过程（第二章）。

8-pCPT-2'-O-Me-cAMP (007)是 cAMP 的一个同源物，它可以特异地激活 Epac，而不影响 PKA 的活性。利用这一人工合成物，研究者们将 Epac 蛋白定位在 cAMP 介入的信号转导途经的一个非常重要的位置上。例如，Epac 已被证明介入了细胞增殖过程；VE-/E-cadherin　介导的细胞间的连结的形成过程；整合蛋白介导的细胞和基质的附着过程；胰岛素的分泌过程以及钠质子交换活性的调控过程。但是，为了了解 Epac 蛋白的更多的功能，我们制备了并鉴定了 Epac 的单克隆和多克隆抗体。由于 5D3，Epac 的单克隆抗体之一，可以识别激活状态的 Epac 蛋白，因此，在第三章我们详细地鉴定了这个单克隆抗体并把它的表抗原位置定位在了 Epac 调控区的 cAMP 结合域内，而第 273 亮氨酸的位置附近则是这一抗体表原位的精细位置，而这一位置正好处于 Epac 蛋白自体结合区。

Epac 的亚细胞定位是本论文的重要部分。而且对 Epac 进行亚细胞定位对于了解 Epac 的功能也很重要。利用我们制备的 Epac 单克隆和多克隆抗体，我们发现 Epac 主要分部在细胞核膜周围，特别是高尔基和内质网上，同时，细胞膜也是 Epac 蛋白的主要的分布区，特别是在极性化细胞的微乳突上（Microvilli）。为了了解对 Epac 亚细胞定位起重要作用的功能域，我们利用一系列的 Epac 缺失突变体发现 Epac 的正确定位需要两个膜定位信号，一个是 DEP 作用域，另一个是 N 末端的 49 个氨基酸。这两个膜定位信号不仅在 Epac 正确定位是起非常重要的作用，而且也是 Epac 激活小 G 蛋白 Rap 所不可缺少的功能区。N 末端的 49 个氨基酸只在 Epac 定位在微乳突上发挥作用，而且仅仅是 49 个氨基酸足以牵引 Epac 蛋白定位到细胞的微乳突上。DEP 作用域尽管在 Epac 定位在微乳突上没有发挥作用，但细胞组分的分离结果表明 DEP 作用域仍然在 Epac 的膜定位过程中发挥一定的功能。利用 Epac 作为诱饵，ERM 家族蛋白 Radixin 和 Ezrin 从酵母双杂库（Y2H）中被筛选出来。Radixin 和 Ezrin 是一个支架蛋白（Scaffold），它们定位在细胞的微乳突上并在连结细胞骨架和极性细胞膜之间起作用。Epac 通过它的 N 末端的 49 个氨基酸和 Radixin 和 Ezrin 的 N 端 结合在一起，但是这一个蛋白复合体的功能还是一个问号。同时在免疫共沉淀的试验中，我们发现 Epac 只能和激活态的 Radixin 和 Ezrin 免疫共沉淀，这一现象预示这些 ERM 蛋白可以调控 Epac 区域性功能的发挥（第四章）。在研究 Epac 蛋白的亚细胞定位过程中，我们发现当 Epac 的 N 末端的 49 个氨基酸缺失后，Epac 常常聚积在细胞核中。这一现象也在 HGF（细胞分散因子）诱导的细胞分散和 RapV12 引起的细胞扩散试验中得到了证实。这一结果预示着细胞极性或者细胞的微乳突结构的破坏可以诱导 Epac 蛋白转移到细胞核中，但是转移到细胞核中的 Epac 将发挥什么样的功能仍然有待于进一步的研究（第四章的 Addendum）。