去棕榈酰化修饰下G蛋白偶联受体激酶6核定位信号鉴定

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摘要 G蛋白偶联受体激酶6(G protein-coupled receptor kinase 6, GRK6)依赖去棕榈酰化修饰及特定核定位序列(nuclear localization sequence, NLS)实现胞核定位, 但NLS的关键基团及其对激酶活性的影响尚不清楚。该研究首先通过构建一系列缺失突变子, 初筛去棕榈酰化条件下GRK6的NLS结构域; 然后采用点突变技术进一步确定NLS结构域中Lys(K)[389]、Lys(K)[390]、Lys(K)[391]3个关键基团; 最后通过检测内源性毒蕈碱M3受体介导的细胞内钙流, 证实NLS突变子对M3受体介导的细胞内钙流信号的抑制作用无明显影响。该研究为进一步揭示GRK6胞核转运机制及其功能提供了有价值的信息。

关键词 GRK6; 去棕榈酰化; 核定位信号; G蛋白偶联受体激酶

Identification of Nuclear Localization Sequence of G Protein-Coupled Receptor Kinase 6 under Depalmitoylation

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Abstract G protein-coupled receptor kinase 6 (GRK6) is capable of achieving nuclear translocation through depalmitoylation. Although a nuclear localization sequence (NLS) region has reported, the key residue(s) in the NLS domain as well as its effect on the kinase activity are not yet clear. In this study, we first verified a predicted NLS domain through screening a series of GRK6 deletion mutants under the condition of depalmitoylation. Then, we further identified three residues, Lys(K)[389], Lys(K)[390] and Lys(K)[391], which respectively played essential role in the nuclear translocalization of GRK6 by using a point mutation technology. Finally, the biological function of the mutants in the M3 receptor signaling, comparing to GRK6 WT, was analyzed using a calcium mobilization assay and found no significant effect on their kinase activities. This study provides valuable information to further study the nuclear translocation mechanism and function of GRK6.

Keywords GRK6; depalmitoylation; nuclear localization sequence; G protein-coupled receptor kinase

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G蛋白偶联受体激酶(G protein-coupled receptor kinases, GRKs)属于丝/苏氨酸激酶家族, 通过磷酸化细胞膜上与配体结合的G蛋白偶联受体(G protein-coupled receptors, GPCRs), 负性调控(减敏)GPCR信号通路, 参与细胞广泛的正常及病理生物学活动。迄今为止, 在哺乳动物细胞中共发现7个GRKs家族成员, 分为3个亚家族: 视紫质激酶亚家族(GRK1/7)、β肾上腺素受体激酶亚家族(GRK2/3)以及GRK4亚家族(GRK4/5/6)[1]。

最新的研究发现, GRKs除了调控GPCR信号通路外, 一些GRKs分子如GRK2、GRK4、GRK5、GRK6能够磷酸化组蛋白去乙酰化酶(HDAC)、NF-κB、p53、DNA-PKCs等重要的胞核内信号分子, 影响靶基因的生物学功能[2-6]。定位于细胞核是GRKs直接调控核内靶分子的前提, 但目前对GRKs的核定位机制仍不清楚。Jiang等[7]发现, GRK6通常主要定位于细胞膜, 但在羧基端的3个半胱氨酸残基(Cys^{561}、Cys^{562}、Cys^{565})去棕榈酰化(depalmitoylation)修饰条件下发生"胞膜–胞质–胞核"穿梭转运和胞核聚集, 而人工突变赖氨酸(Lys^{219})则抑制其胞核转运。Johnson等[8]发现, 突变GRK6分子^{388}RKKKIKRE^{395}中的Lys残基导致其核定位丧失, 其详细机制有待进一步明确。本研究在我们前期建立的去棕榈酰化修饰下GRK6胞核聚集的细胞模型上, 通过构建系列缺失突变子, 证实^{384}PFQQRKKK^{391}是GRK6实现核定位功能必需的结构域, 并在此基础上进一步鉴定了此核定位序列(nuclear localization sequence, NLS)中的关键调控基团及其对GRK6分子激酶活性的影响。该研究为揭示GRK6的胞核转运机制及其潜在的核内生物学功能提供了有价值的信息。

1 材料与方法

1.1 主要试剂与仪器

主要试剂: 二甲基亚砜(DMSO)(美国Sigma公司)、卡巴可(美国Med Chem Express公司)、兔抗人GRK6抗体和兔抗人SP1抗体(美国Santa Cruz公司)、兔抗人Caspase 3抗体(美国Cell Signaling Tech公司)、ECL试剂(Pierce, 美国赛默飞世尔科技公司)、FLUOFORTE Calcium Assay Kit(瑞士Enzo公司)。主要仪器: Tecan F-500酶标仪(瑞士Tecan公司)、Z2正置荧光显微镜(德国Zeiss公司)、Optima MAX-XP超速离心机(美国Beckman公司)、CO_2细胞培养箱(美国NBS公司)。pcDNA3-GRK6、pcDNA3-GRK6/3S pEGFP(N1)质粒由Wedegaertner教授(美国Thomas Jefferson大学医学院生物化学与分子生物学系)赠送。

1.2 细胞培养

人胚肾HEK293细胞购自中国科学院上海细胞库, 常规培养于体积分数10%胎牛血清(杭州四季青生物工程材料有限公司公司)的DMEM(美国Gibco公司)培养液、含5% CO_2的37 ℃培养箱中, 每3~4天换液传代。

1.3 缺失及点突变子构建

GRK6缺失或单点突变均通过两步法PCR方法获得。第一步, 以GRK6或GRK6/3S为模板, 通过两对引物(引物序列见表1, 引物名后缀a和b配对, c和d配对)PCR扩增获得含部分重叠序列的两个片段A和B; 第二步, 以产物片段A和B为模板, 用后缀为a和d(以引物名后缀为a和d1装入pEGFP-N1, 后缀为a和d2装入pcDNA3)的一对引物PCR扩增获得突变序列并通过双酶切装入pcDNA3或pEGFP-N1载体。

1.4 免疫印迹实验

蛋白样品加入5×上样缓冲液, 充分混匀后沸水中变性5 min, SDS-PAGE凝胶电泳, Bio-Rad湿转装置将蛋白质转移至0.45 μm的PVDF膜; 5%脱脂奶粉溶于TBS-T室温封闭1 h, 一抗4 ℃过夜孵育。TBS-T洗3次, 每次5 min。HRP标记的二抗1:5 000稀释后室温孵育0.5 h。TBS-T洗3次, 每次5 min。ECL试剂显色, 使用感光胶片检测信号。

1.5 免疫荧光染色

固定液(0.25%戊二醛溶于PBS)固定5 min, PBS洗2次, 穿孔缓冲液(1% Triton X-100溶于TBS)润洗1次, 封闭液(5%脱脂奶粉溶于穿孔液)封闭30 min, 一抗室温孵育1 h, 穿孔缓冲液洗3次, 二抗避光室温孵育30 min, 穿孔缓冲液洗3次, DAPI染液室温孵育5 min, 抗淬灭剂封片。

1.6 细胞影像分析

蔡司显微镜操作系统Zeiss AxioVision 4.X获取图像后, 每组随机选10个细胞, 测量胞核和胞质的平均荧光强度, 计算胞核与胞质平均荧光强度的比值。

1.7 细胞组分分离

10 cm培养皿的细胞加入0.5 mL预冷低渗液(10 mmol/L KCl、20 mmmol/L HEPES、1 mmol/L EDTA、1.5 mmol/L $MgCl_2$、250 mmol/L Sucrose、

表1 引入缺失或点突变的PCR引物

Table 1 Primers list for PCR

引物 Primers	序列(5′→3′) Sequences (5′→3′)
6-Hind III-F-a	tca agc tta tgg agc tcg aga aca tcg tag
6-Δ94~100-b	ccg ccc aca ggt cac ttc ata ctc ggc c
6-Δ94~100-c	gaa gtg acc tgt ggg cgg cag cta acg c
6-Δ133~169-b	tgc agg aaa cgc agc cgc tgg gtg cag ttc gtc
6-Δ133~169-c	cca gcg gct gcg ttt cct gca gtg gaa gtg g
6-Δ384~391-b	ccg ctt gat cga ctg gcc tgc gat cat c
6-Δ384~391-c	ggc cag tcg atc aag cgg gag gag gtg ga
6-Δ516~545-b	tta gga ggt gcc acc atc tcg ttc tgc cag gg
6-Δ516~545-c	cga gat ggt ggc acc tcc taa aaa ggg act g
6-388R/A-R-b	tct tct tcg cct gct gga agg gcg act g
6-388R/A-F-c	tcc agc agg cga aga aga aga tca agc g
6-389K/A-R-b	gat ctt ctt cgc cct ctg ctg gaa ggg cga
6-389K/A-F-c	cag agg gcg aag aag atc aag cgg gag
6-390K/A-R-b	ttg atc ttc gcc ttc ctc tgc tgg aag gg
6-390K/A-F-c	gag gaa ggc gaa gat caa gcg gga gga g
6-391K/A-R-b	cgc ttg atc gcc ttc ttc ctc tgc tgg aag
6-391K/A-F-c	gaa gaa ggc gat caa gcg gga gga ggt g
6-BamHI-R-d1	tgg atc ccg gag gcg ggt ggg gag ctc ttc ct
6-BamHI-R-d2	att gga tcc cta gag gcg ggt ggg gag ct

1 mmol/L EGTA、1 mmol/L DTT、1 mmol/L PMSF), 刮取细胞并转移至1.5 mL离心管中, 1 mL注射器(25 Ga针头)抽吸10次, 冰上静置20 min。重悬后取0.4 mL悬液3 000 r/min于4 ℃离心5 min, 所得沉淀用于制备细胞核组分, 上清则进一步制备胞质组分。胞核组分制备: 前述所得沉淀加入0.4 mL低渗液重悬沉淀, 1 mL注射器抽吸10次, 3 000 r/min于4 ℃离心5 min。取沉淀为细胞核组分, TBS-T洗3次后将沉淀溶于0.4 mL裂解液(RIPA)。细胞质组分制备: 将前述上清液40 000 r/min于4 ℃离心30 min, 取上清液为胞质组分。

1.8 钙离子移动实验

HEK293细胞分别转染pcDNA3-GRK6 WT(野生型)、pcDNA3-GRK6 K389A、pcDNA3-GRK6 K390A、pcDNA3-GRK6 K391A。于含G418(0.5 g/L)抗性完全培养基中筛选, 抗GRK6抗体行蛋白免疫印迹及免疫荧光染色检测鉴定阳性克隆, 扩培并冻存待用。分别将稳定表达GRK6 WT、GRK6 K389A、GRK6 K390A、GRK6 K391A的细胞种植于96孔板, 24 h后移除培养基加入染液(100 μL/孔, 按照检测试剂盒说明书配制), 37 ℃生化培养箱中避光静置45 min, 室温避光静置15 min。酶标仪的钙流检测程序设定: 间隔1 s, 100个循环; 激动剂卡巴可加入动作时间点为第21个循环, 浓度为10 μmol/L, EX为492 nm, EM为535(20) nm。

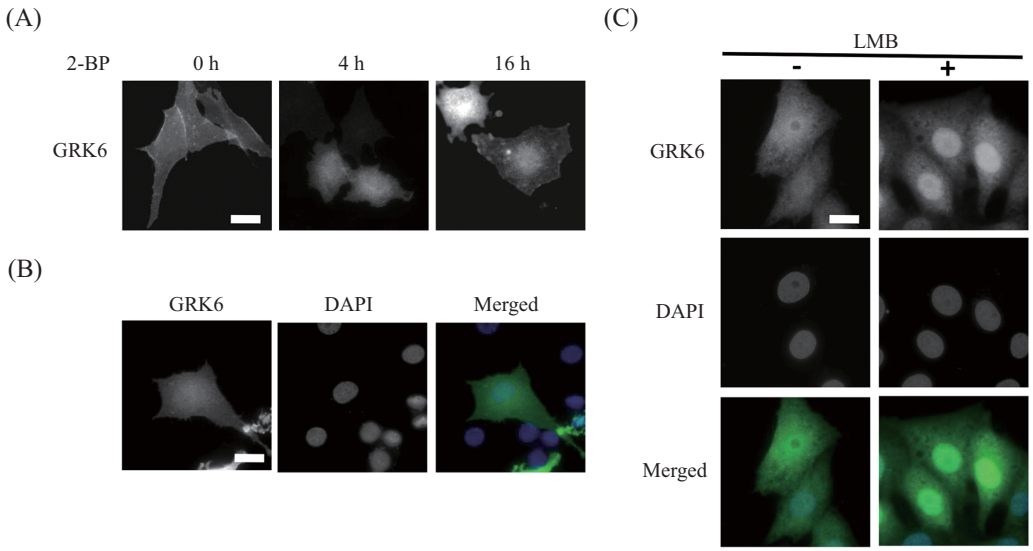
1.9 统计学分析

采用SPSS 20.0统计学软件, 结果以均数±标准差表示, Student's *t*-test分析其统计学意义, 当$P<0.05$时, 判定具有显著性差异。

2 结果

2.1 去棕榈酰化修饰促进GRK6胞核转运

pcDNA3-GRK6质粒转染HEK293细胞48 h, 分别于0 h、4 h和16 h以棕榈酰化抑制剂2-溴十六烷酸(2-bromopalmitate, 2-BP)处理细胞。抗GRK6抗体免疫荧光染色, 荧光显微镜下观察GRK6分子的亚细胞定位。2-BP处理细胞后, GRK6由胞膜转运至胞质并呈现显著的胞核聚集(图1A); 将GRK6羧基端的三个棕榈酰化修饰潜在基团Cys^{561}、Cys^{562}和Cys^{565}替换成丝氨酸(Ser, 3S), GRK6/3S突变子呈现显著的胞核聚集(图1B); 出核抑制剂Leptomycin B(LMB)处理显著增强其细胞核聚集(图1C)。这些



A: pcDNA3-GRK6质粒瞬时转染HEK293细胞48 h, 于0 h、4 h、16 h用100 μmol/L浓度的2-BP处理细胞。抗GRK6抗体免疫荧光染色, 荧光显微镜观察GRK6亚细胞定位。标尺=10 μm。B: 棕榈酰化缺陷型pcDNA3-GRK6/3S质粒转染HEK293细胞, 24 h后以抗GRK6抗体行免疫荧光染色, DAPI染核, 荧光显微镜观察GRK6亚细胞定位。标尺=10 μm。C: pcDNA3-GRK6/3S质粒转染HEK293细胞, 24 h后以20 μg/L Leptomycin B(LMB)处理细胞8 h, 抗GRK6抗体免疫荧光染色, DAPI染核, 荧光显微镜观察GRK6亚细胞定位。GRK6/3S突变子系指以丝氨酸(S)替代野生型GRK6分子561、562和565位点的半胱氨酸, 标尺=10 μm。

A: HEK293 cells were transiently transfected with GRK6 expression vectors. Cells were treated with 100 μmol/L 2-bromopalmitate(2-BP) for 0 h, 4 h and 16 h before fixing the cells 48 h after transfection. Localization of GRK6 were visualized by immunofluorescence staining using the anti-GRK6 antibody. Bar=10 μm. B: HEK293 cells were transiently transfected with GRK6/3S expression vectors. GRK6/3S were visualized by immunofluorescence staining using the anti-GRK6 antibody, and nuclei were visualized with DAPI staining. Bar=10 μm. C: HEK293 cells were transfected with pcDNA3-GRK6/3S plasmid, after 24 h, cells were treated with 20 μg/L Leptomycin B(LMB) for 8 h. Localization of GRK6 were visualized by immunofluorescence staining using the anti-GRK6 antibody. The GRK6/3S mutation of GRK6 indicates substitution of cysteine acids 561, 562 and 565 with serine, bar=10 μm.

图1 去棕榈酰化修饰促进GRK6胞核转运

Fig.1 Depalmitoylation of GRK6 promotes its nuclear translocation

结果表明, 去棕榈酰化修饰促进GRK6胞膜–胞核穿梭转运。

2.2 ^{384}PFQQRKKK^{391}为GRK6胞核定位所必需的序列

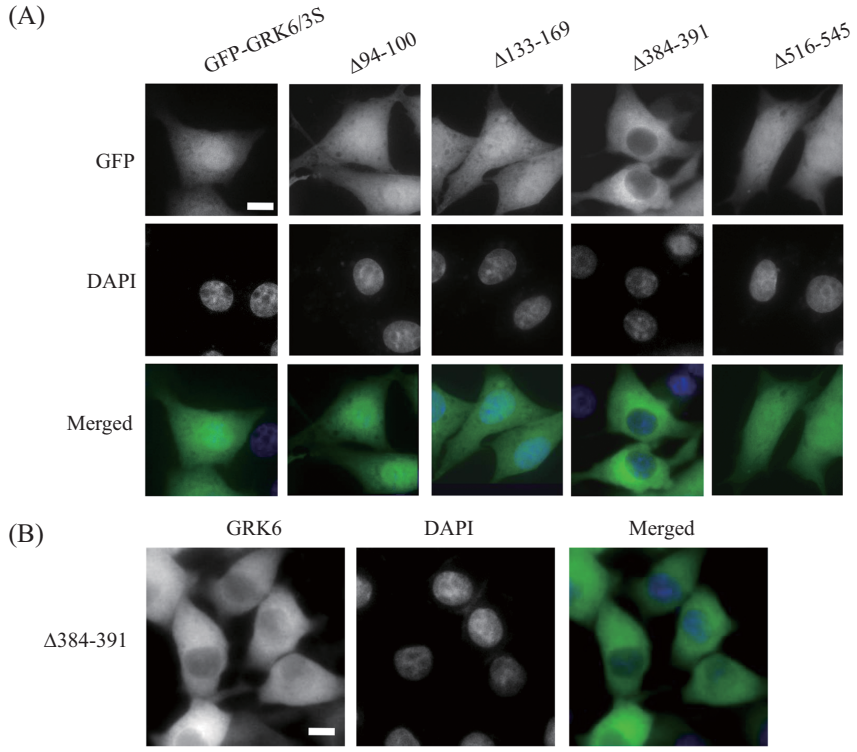
为研究GRK6脱棕榈酰化依赖的胞核转运, 我们首先采用PredictNLS软件(哥伦比亚大学生物信息学中心)预测分析获得多个GRK6分子的潜在NLS。据此信息, 我们以GRK6/3分子作为模板, 采用两步法PCR扩增构建了4个融合GFP荧光蛋白标签的潜在NLS缺失突变子, 分别转染HEK293细胞, 24 h后荧光显微镜下观察, 与GRK6/3S相比, ^{384}PFQQRKKK^{391}缺失突变子(GFP-GRK6/3S Δ384~391)显著失去核定位能力(图2A和图2B), 提示该序列可能是GRK6分子核定位的关键结构域。

2.3 潜在NLS中关键基团的鉴定

为进一步确定去棕榈酰化修饰下GRK6核定位的关键基团, 我们以GRK6/3S为模板, 将上述潜在NLS中的R^{388}/K^{389}/K^{390}/K^{391}分别替换成丙氨酸(A), 转染HEK293细胞, 24 h后分别行免疫荧光显微镜术观察, 发现该4个碱性氨基酸突变均不同程度地影响GRK6/3S分子的胞核聚集(图3A)。荧光强度定量分析和细胞组分免疫印迹术分析结果显示, 突变K^{389}、K^{390}或K^{391}后, GRK6胞核/胞质分布的比值显著下降(P<0.05)(图3B和3C)。

2.4 NLS基团突变对GRK6激酶活性的影响

GRKs通过磷酸化细胞膜上与配体结合的GPCRs, 减敏GPCRs介导的跨膜信号转导。另一方面, GRKs能够磷酸化一些重要的胞核内信号分子, 调控靶基因的生物学功能。为了探究上述GRK6 NLS突变对激酶活性的潜在影响, 我们将稳定表达GRK6 WT(野生型)或GRK6 K389A、GRK6 K390A、GRK6 K391A的HEK293细胞种于96孔板, 24 h后以M3受体的激动剂卡巴可(10 μmol/L)刺激细胞, 分别动态检测钙离子移动信号[9]。结果显示, 与GRK6 WT相似, K^{389}、K^{390} 或 K^{391}位点突变子对M3受体介导的钙流具有显著的抑制作用(P<0.01), 见图4。



A: pEGFP-GRK6/3S及其缺失突变子(Δ94~100、Δ133~169、Δ384~391、Δ516~545)质粒分别转染HEK293细胞, 24 h后DAPI染核, 荧光显微镜观察GRK6的亚细胞定位。标尺=10 μm。B: pcDNA3-GRK6/3S Δ384~391质粒转染HEK293细胞, 24 h后以抗GRK6抗体行免疫荧光染色, DAPI染核, 荧光显微镜观察GRK6的亚细胞定位。标尺=10 μm。

A: HEK293 cells were transfected with GFP-GRK6/3S or its deletion mutants (Δ94-100、Δ133-169、Δ384-391、Δ516-545) as indicated. 24 h after transfection, localization of GRK6 were visualized by microscopy and nuclei were visualized with DAPI staining. Bar=10 μm. B: HEK293 cells were transfected with pcDNA3-GRK6/3S deletion mutant (Δ384-391). 24 h after transfection, localization of GRK6 were visualized by immunofluorescence staining using the anti-GRK6 antibody and nuclei were visualized with DAPI staining. Bar=10 μm.

图2 GRK6依赖于^{384}PFQQRKKK^{391}序列实现胞核定位

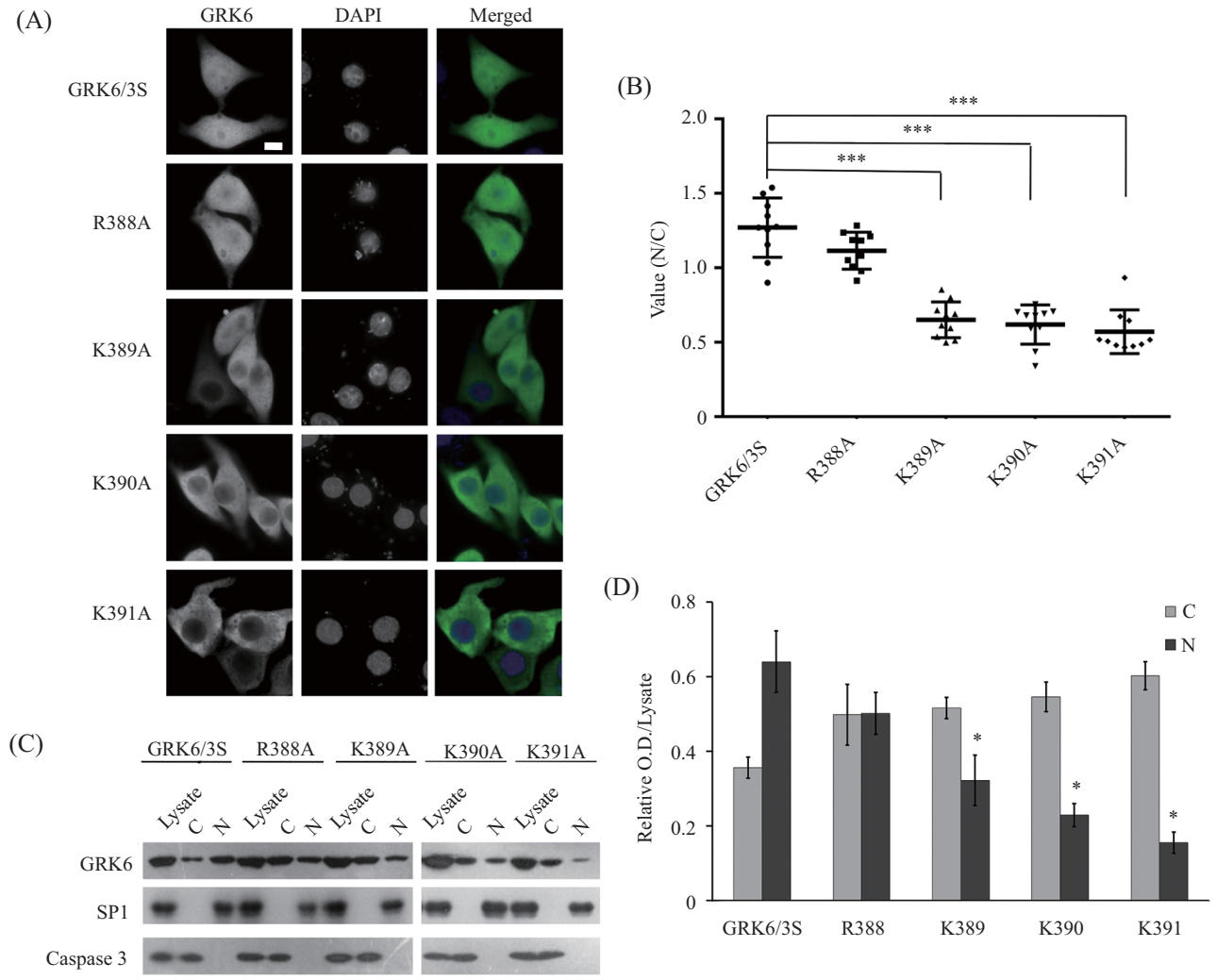
Fig.2 Nuclear translocation of GRK6 relies on its ^{384}PFQQRKKK^{391} sequence

3 讨论

GRKs通过磷酸化细胞膜上的GPCRs参与神经传导、心血管和呼吸道平滑肌调节、水钠平衡、激素代谢等十分广泛的生理活动, 是细胞正常生命活动的重要调节者和新药研发的重要靶点[1]。新近发现, 某些GRK分子(如GRK2、GRK4、GRK5和GRK6)等还通过非GPCR磷酸化作用参与组蛋白去乙酰化酶(HDAC)、NF-κB、p53等核内重要生物分子的功能调控[2-5]。目前对GRKs的胞核定位机制所知甚少。Johnson等[7]发现, GRK5、GRK6含有一个^{388}RKxKxKRE^{395}的潜在NLS共同序列, 删除GRK5-^{388}RKEKVKRE^{395}或GRK6-^{388}RKKKIKRE^{395}序列或突变其中的全部碱性氨基酸残基导致它们核定位丧失。Jiang等[6]发现, GRK6可以通过羧基端的3个半胱氨酸残基(Cys)的可逆性棕榈酰化修饰实现“胞膜–胞质–胞核”穿梭转运调控, 出核抑制剂LMB增强其细胞核聚集, 人工突变Lys(K)^{219}则抑制其胞核转运。这些研究表明, GRK6分子存在复杂的胞核转运调控机制。

本研究通过PredictNLS软件预测、并以去棕榈酰化修饰缺陷的GRK6分子为模板构建一系列的缺失突变子, 证实^{384}PFQQRKKK^{391}是其胞核定位所必需的结构, 与已报道的序列部分重叠。我们进一步在GRK6去棕榈酰化条件下逐一突变该NLS中的4个碱性氨基酸残基, 发现它们均能够减弱GRK6的胞核定位, 以K^{389}、K^{390}和K^{391}为显著, 提示这些基团对GRK6的核定位具有单独影响作用, 拓展了我们对GRK6核定位调控元件的认识。

磷酸化受体或其他底物分子是GRKs的重要生物学功能。此前有关GRK6 NLS的研究报道包括对^{388}RKKKIKRE^{395}序列的鉴定研究, 并无其对激酶活性影响的观察。本研究发现, 突变K^{389}、K^{390}或K^{391}



A: pcDNA3-GRK6/3S及其单点突变子(R388A、K389A、K390A、K391A)质粒分别转染HEK293细胞, 24 h后以抗GRK6抗体行免疫荧光染色, DAPI染核, 荧光显微镜观察GRK6的亚细胞定位。标尺=10 μm。B: 镜下随机选取10个转染细胞, Zeiss AxioVision 4.X软件测定胞核(N)和胞质(C)荧光强度并计算比值, ***P<0.001。C: pcDNA3-GRK6/3S及其单点突变子(R388A、K389A、K390A、K391A)质粒分别转染HEK293细胞, 24 h后收集细胞, 分离细胞质(C)及细胞核(N)细胞组分以抗GRK6抗体行蛋白免疫印迹检测GRK6分布; SP1及Caspase 3分别为胞核与胞质组分的内参。D: 3次独立蛋白印迹蛋白信号条带灰度定量的统计学分析, 以细胞总裂解液(lysate)中GRK6水平标化。N: 胞核组分; C: 胞质组分, *P<0.05, 与GRK6/3S细胞组比较。

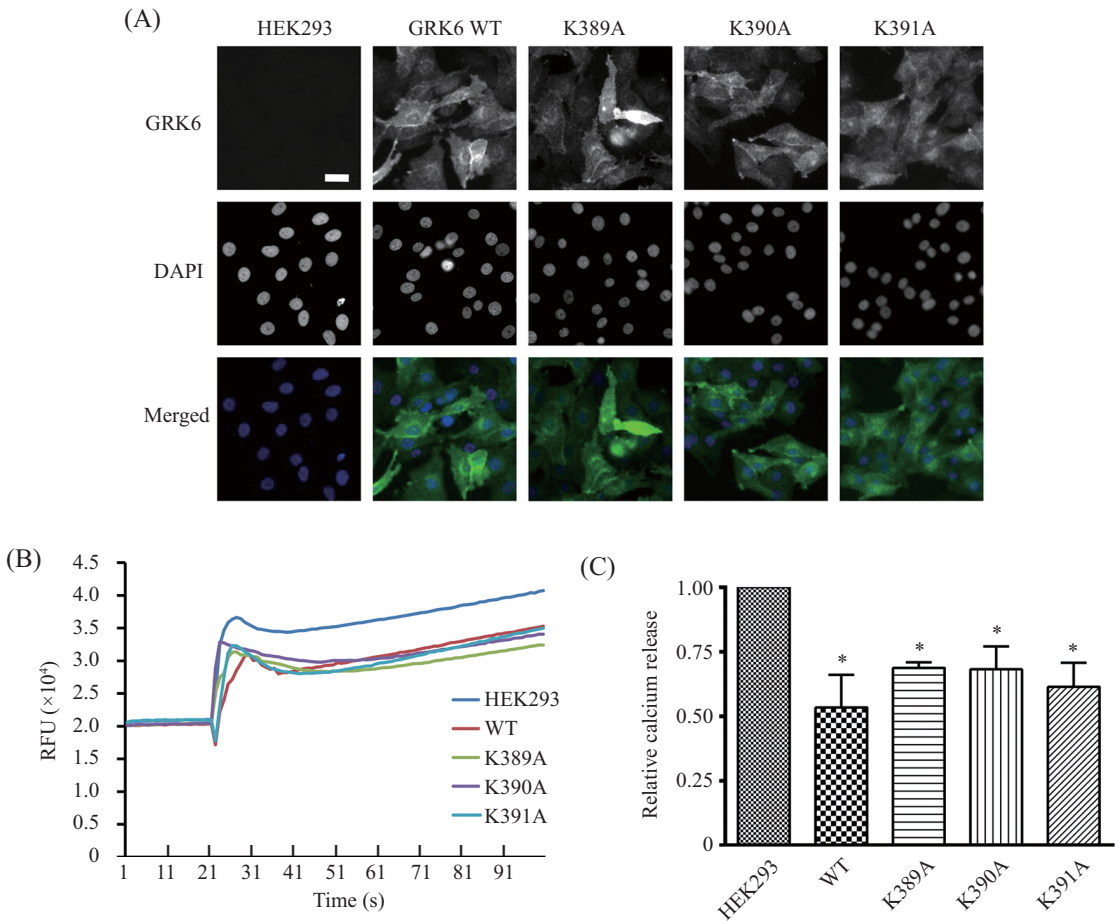
A: HEK293 cells were transfected with GFP-GRK6/3S or its deletion mutants (R388A、K389A、K390A、K391A) as indicated. Localization of GRK6 were visualized by immunofluorescence staining using the anti-GRK6 antibody and nuclei were visualized with DAPI staining. Bar=10 μm. B: 10 cells were analyzed randomly per group using Zeiss AxioVision 4.X software with the intensity of the nuclear (N) or cytoplasmic (C) regions, ***P<0.001. C: HEK293 cells were transiently transfected with expression vectors for GRK6/3S or the indicated mutants. 24 h after transfection, cells were lysed, and the lysates were separated into cytosolic (C) and nuclear (N) fraction. The expressed proteins were detected by Western blot with the anti-GRK6 antibody. SP1 and Caspase 3 are shown as loading controls of cytosolic and nuclear fraction, respectively. D: quantitative data of the protein levels of GRK6 in Western blot, which are normalized by total cell lysates. N: nuclear fraction; C: cytoplasmic fraction, *P<0.05 *vs* GRK6/3S cells group.

图3 潜在NLS中关键基团的鉴定

Fig.3 Identification of essential residues of the putative NLS

的GRK6分子对M3受体介导的钙流仍具有显著的抑制作用, 表明这些NLS位点不影响激酶活性。此结果为开展GRK6的功能性核定位研究提供了依据。

综上, 本研究对GRK6的核定位信号关键基团进行了鉴定, 这些基团的突变不影响其激酶活性, 为进一步揭示GRK6胞核转运机制及其功能提供了有价值的信息。



A: 免疫荧光染色检测GRK6 WT或突变子(GRK6K389A、GRK6K390A和GRK6K391A)稳定转染的HEK293细胞的GRK6表达情况。标尺=20 μm。B: 钙离子移动实验检测稳定表达GRK6 野生型(WT)或突变子(K389A、K390A和K391A)的HEK293细胞对内源性M3受体介导细胞内钙离子释放的影响。激动剂卡巴终浓度: 10 μmol/L; RFU: 相对荧光强度单位。C: 三次独立钙离子移动实验的细胞释放钙离子峰值的统计学分析。*P<0.05, 与HEK293细胞组比较。

A: detection of GRK6 expression by immunofluorescence staining in HEK293 cells stably expressing the GRK6 WT or its mutants of K389A, K390A and K391A. Bar=10 μm. B: calcium flux assay for detection of intracellular calcium mobilization induced by endogenous M3 receptor in HEK293 cells that stably expresses GRK6 WT or K389A, K390A and K391A mutants working concentration of agonist carbachol: 10 μmol/L; RFU: relative fluorescence units. C: quantitative data of cellular calcium release in calcium mobilization assay. *P<0.05 *vs* HEK293 cells group.

图4 GRK6 NLS基团对其激酶活性的影响

Fig.4 Effect of essential residues of the GRK6 NLS on its kinase activity

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