黄色荧光蛋白的结构对Annexin A5凋亡检测功能的影响探究

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摘要 细胞凋亡早期, 磷脂酰丝氨酸(PS)从细胞膜内侧翻转到细胞膜外侧, Annexin A5可与PS高亲和力结合, 这是其检测凋亡的原理。绿色荧光探针标记的Annexin A5和碘化丙啶联合标记应用于流式细胞术进行细胞凋亡检测, 该方法灵敏度高、特异性好、效率高, 已成为目前全世界通用的细胞凋亡检测方法。除FITC-Annexin A5外, Annexin A5-EGFP融合蛋白是另一个常用的简单、可信、易于制备的凋亡检测探针。黄色荧光蛋白是绿色荧光蛋白的一种突变体, 其荧光向红色光谱偏移。目前, 有三种改良的黄色荧光蛋白: Citrine、Venus、Ypet, 这三种改良的蛋白荧光更亮、更稳定、成熟更快。该文选择Citrine、Venus、Ypet来制备Annexin A5凋亡检测探针, 通过原核表达和分离纯化Annexin A5-Citrine、Annexin A5-Venus和Annexin A5-Ypet融合蛋白, 获得了高产量的可溶性蛋白, 探究这三种融合蛋白与凋亡细胞的结合能力, 筛选出适用于流式检测的黄色荧光蛋白标记的Annexin A5, 为后续研究奠定基础。以pET28a-Annexin A5-EGFP-his重组质粒为模板, 利用分子生物学手段又构建了重组质粒pET28a-Annexin A5-Citrine-his、pET28a-Annexin A5-Venus -his、pET28a-Annexin A5-Ypet-his, 然后通过Ni柱亲和层析纯化获得了这三种蛋白, 纯度约为90%。该文重点比较了三者检测细胞凋亡的能力, 流式细胞仪检测结果显示, Annexin A5-Citrine、Annexin A5-Venus和Annexin A5-Ypet融合蛋白均可以识别和标记凋亡细胞, 它们与凋亡细胞的亲和力分别是3 113.0 nmol/L、444.3 nmol/L和391.6 nmol/L。三者与凋亡细胞的亲和力差异很大, 通过对Citrine、Venus、Ypet的氨基酸序列进行分析, 该文初步发现了决定三个黄色荧光蛋白与凋亡细胞亲和力的关键氨基酸。

关键词 膜联蛋白A5; 纯化; 凋亡检测; 流式细胞术; 序列比对

The Influence of Structrue of Yellow Fluorescent Proteins on the Apoptotic Detection Ability of Annexin A5

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Abstract At the early stage of apoptosis, phosphatidylserine (PS) will turn over from the inner cell membrane to the outer cell membrane. When calcium ions are present, Annexin A5 can combine to PS at high affinity and this is the principle of apoptosis detection. Apoptosis detection based on Annexin A5 labeled with green fluorescent probes/iodide by FCM (flow cytometry) is sensitive, efficient and specific. Annexin A5-EGFP fusion protein is a simple, reliable apoptosis detection probe and it is easy to prepare. Yellow fluorescent protein is a mutant of green fluorescent protein and its fluorescence spectra shifts to red spectra. Currently, there are three modified yellow fluorescent proteins: Citrine, Venus, Ypet and the three modified proteins' fluorescence are brighter, more stable and mature faster. In this paper, Citrine, Venus and Ypet were selected to prepare Annexin A5 apoptosis detection probes. Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet were expressed in the prokaryotic expression system with a high yield of soluble protein. Also, we explored the combination ability of the three fusion proteins and apoptotic cells and then screened out Annexin A5 labeled by yellow fluorescent proteins, which was suitable for apoptosis detection based on FCM in order to lay the foundation for further study. Three new recombinant plasmids pET28a-Annexin A5-Citrine-his, pET28a-Annexin A5-Venus-his, pET28a-Annexin A5-Ypet-his were constructed for the expression. Three recombinant proteins were purified by the Ni column affinity chromatography and their purity is about 90%. Also, this paper compared the three fusion proteins' ability to detect apoptosis and flow cytometric analysis showed that Annexin A5-Citrine, Annexin A5-Venus, Annexin A5-Ypet could recognize and bind to apoptotic cells and their affinity with apoptotic cells was respectively 3 113.0 nmol/L, 444.3 nmol/L, 391.6 nmol/L. The affinity between the three fusion proteins and apoptotic cells was very different. The paper analyzed the amino acid sequence of Citrine, Venus and Ypet and preliminarily found the key amino acids, which determine the affinity of Annexin A5 marked with yellow fluorescent proteins and apoptotic cells.

Keywords Annexin A5; purification; apoptosis detection; FCM (flow cytometry); sequence alignment

细胞凋亡是细胞程序性死亡最常见的一种形式, 它表现为细胞收缩、染色质固缩、细胞膜出泡、细胞凋亡蛋白酶caspase的激活以及细胞膜表面外翻的磷脂酰丝氨酸呈现出“吞噬”的信号[1]。凋亡细胞的清除是通过吞噬细胞完成的, 吞噬细胞可以识别死亡细胞的上述表型[2]。检测细胞凋亡的方法大多是基于这些形态学的变化和生化指标。

目前, 应用最为广泛的凋亡检测探针是Annexin A5。基于Annexin A5的凋亡检测方法是将Annexin A5联结上生物素、荧光素或放射性配体, 使之成为一种可检测的标记物[3-5], 在钙离子存在的情况下, 将凋亡细胞与Annexin A5复合物进行混合, 凋亡细胞就会迅速结合带有标记的Annexin A5。这种结合可以通过多种技术来测量, 如流式细胞术、激光扫描细胞术、共聚焦激光扫描显微术等[6-8]。为了满足不同实验的需要, 各种标记的Annexin A5探针相继被开发[9-10], 且相关产品也实现了商品化。

化学偶联方法标记的Annexin A5被广泛使用, 如最常用的FITC(异硫氰酸荧光素)标记的Annexin A5。该探针的制备过程包括蛋白的表达纯化、化学偶联荧光素以及后续游离荧光素的去除, 这一过程涉及多步操作并需要精确控制[11-15], 非常复杂繁琐; 另一方面, 化学标记Annexin A5的最终产物通常是不同标记程度的混合物, 非常不均一。而荧光蛋白标记的Annexin A5相对均一, 且荧光强度高、光稳定性强[12-16]。而同样是绿色荧光的Annexin A5-EGFP荧光蛋白融合分子探针则不涉及化学偶联方法中的多步操作, 只需要对融合蛋白进行原核表达和分离纯化, 操作简便且成本低, 极具应用价值。

黄色荧光蛋白作为绿色荧光蛋白的一种变体, 它也广泛应用于细胞生物学和分子生物学领域。黄色荧光蛋白最早的变体是EYFP[17], 目前它仍被广泛使用, 但其pKa值高, 对卤化物敏感, 其应用并不理想。单体形式的变体柠檬黄Citrine[18]和Venus[19]是目前应用最多的黄色荧光蛋白探针。另一种很有应用潜力的黄色荧光蛋白是能量转移黄色荧光蛋白(yellow fluorescent protein for energy transfer, Ypet)[20],

它是已经开发的亮度最强的黄色荧光蛋白, 有很好的光稳定性, 对酸性环境的耐受性要比Venus及其他黄色荧光蛋白变体强。本文将Annexin A5与目前常用的三种黄色荧光蛋白变体Citrine、Venus和Ypet融合表达来制备凋亡检测探针, 以期望获得适合于流式细胞术检测的黄色荧光蛋白标记的Annexin A5探针, 作为绿色荧光标记的Annexin A5的替代检测探针, 以拓宽细胞凋亡检测探针的应用光谱范围。

1 材料与方法

1.1 质粒、菌株和细胞株

pET28a空载质粒、pET28a-Annexin A5-EGFP-his由本实验室保存。pET28a-Annexin A5-Citrine-his、pET28a-Annexin A5-Venus-his、pET28a-Annexin A5-Ypet-his均以pET28a-Annexin A5-EGFP-his为模板重新构建。Top10、BL21菌株保存于本实验室中。Jurkat淋巴瘤细胞由本实验室保存。依托泊苷购自江苏凯基生物技术股份有限公司。

1.2 方法

1.2.1 引物设计 根据*Citrine*、*Venus*、*Ypet*的cDNA序列设计特异性引物, 上下游引物中引入*Nde* I和*Xho* I酶切位点, 具体引物序列如表1所示。PCR扩增条件为: 94 ℃预变性5 min; 94 ℃变性30 s, 60 ℃退火30 s, 72 ℃延伸1 min, 28个循环; 72 ℃延伸7 min。

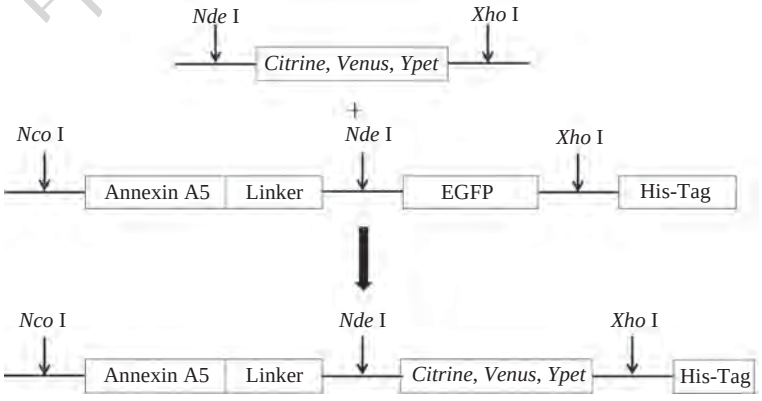
1.2.2 表达载体的构建 将全长的*Citrine*、*Venus*、*Ypet*基因片段和本实验室保存的pET28a-Annexin A5-EGFP-his重组质粒经*Nde* I和*Xho* I双酶切并纯化后进行连接, 使得Citrine、Venus、Ypet的N末端和Annexin A5的C末端通过Linker蛋白连接, 获得了重组质粒pET28a-Annexin A5-Citrine-his、pET28a-Annexin A5-Venus-his、pET28a-Annexin A5-Ypet-his(图1)。重组质粒转化Top10感受态菌株, 涂布卡那霉素抗性平板筛选阳性克隆, 菌落PCR鉴定后, 送至南京金斯瑞生物科技有限公司进行测序。

1.2.3 目的蛋白的表达纯化 pET28a空载质粒、

表1 Citrine、Venus、Ypet的PCR引物

Table 1 PCR primers of Citrine, Venus and Ypet

引物 Primer	序列(5′→3′) Sequences (5′→3′)
Citrine P1	GGA ATC CCA TAT GGT GAG CAA GGG CGA GGA GCT
Citrine P2	CGC TCG AGC TTG TAC AGC TCG TCC AT
Venus P1	GGA ATC CCA TAT GGT GAG CAA GGG CGA GGA GCT
Venus P2	CGC TCG AGC TTG TAC AGC TCG TCC AT
Ypet P1	GGA ATC CCA TAT GAG CAA AGG CGA A
Ypet P2	CCG CTC GAG CTT ATA GAG CTC GTT



将*Citrine*、*Venus*、*Ypet*基因序列插入到pET28a-Annexin A5-EGFP的*Nde* I和*Xho* I位点之间, 构建重组质粒pET28a-Annexin A5-Citrine、pET28a-Annexin A5-Venus、pET28a-Annexin A5-Ypet。

The expression plasmids pET28a-Annexin A5-Citrine, pET28a-Annexin A5-Venus and pET28a-Annexin A5-Ypet were constructed by insertion of *Citrine*, *Venus*, *Ypet* encoding sequence between *Nde* I and *Xho* I sites of pET28a-Annexin A5-EGFP.

图1 pET28a-Annexin A5-Citrine、pET28a-Annexin A5-Venus、pET28a-Annexin A5-Ypet表达质粒的构建

Fig.1 Construction of the pET28a-Annexin A5-Citrine, pET28a-Annexin A5-Venus and pET28a-Annexin A5-Ypet expression plasmids

pET28a-Annexin A5-Citrine-his、pET28a-Annexin A5-Venus-his、pET28a-Annexin A5-Ypet-his重组质粒分别转化大肠杆菌BL21(DE3)感受态细胞, 分别挑取单克隆并接种到3 mL LB液体培养基中(含有50 μg/mL卡那霉素), 220 r/min、37 ℃振荡培养过夜后, 将含有pET28a空载质粒、pET28a-Annexin A5-Citrine-his、pET28a-Annexin A5-Venus-his、pET28a-Annexin A5-Ypet-his重组质粒的过夜菌按1:50的比例转接到3 mL LB液体培养基中(含有50 μg/mL卡那霉素)。220 r/min、37 ℃振荡培养至D_{600}值约为0.6时, 加入异丙基-β-D-硫代半乳糖苷诱导剂(isopropyl-β-D-thiogalactoside, IPTG)至终浓度为1 mmol/L, 分别在37 ℃和20 ℃条件下培养6 h和16 h。诱导完成后, 8 000 r/min离心3 min收集菌体, 然后用PBS缓冲液重悬菌体, 对菌体重悬液进行超声, 超声结束后, 对样品进行SDS-PAGE, 观察其表达情况和存在形式。

由于目的蛋白在20 ℃诱导时主要存在于上清中, 为了纯化方便, 本文选择20 ℃作为目的蛋白的最适诱导温度。将3 mL含有重组质粒的过夜活化菌转接至400 mL LB液体培养基中(含有50 μg/mL卡那霉素), 220 r/min、37 ℃振荡培养3~4 h, D_{600}值达到0.6后, 加入IPTG至终浓度为1 mmol/L, 20 ℃培养16 h。6 000 r/min离心5 min收集菌体, 用40 mL 20 mmol/L Tris缓冲液(含250 mmol/L NaCl)重悬菌体, 在冰浴条件下超声破碎菌体(超声条件为: 超声4 s/间隔8 s, 总共40 min)。超声结束后, 菌液在4 ℃条件下, 16 000 r/min离心20 min, 收集上清。

使用NTA-Ni亲和层析进行蛋白纯化, 镍柱使用前先用20 mmol/L Tris缓冲液(含250 mmol/L NaCl)进行平衡, 平衡体积约为填料体积的5~10倍。上述得到的上清经0.22 μm滤膜过滤后上样。上样完毕后, 用20 mmol/L Tris缓冲液(含250 mmol/L NaCl、50 mmol/L咪唑)清洗未能结合在镍柱上的杂蛋白, 然后用20 mmol/L Tris缓冲液(含250 mmol/L NaCl、250 mmol/L咪唑)洗脱目的蛋白, 收集最大吸收峰处的洗脱液。Ni柱洗脱收集液中含有高浓度咪唑, 使用4 ℃透析法除去咪唑。用含不同浓度咪唑的20 mmol/L Tris缓冲液(含30 mmol/L NaCl)作为透析外液梯度透析目的蛋白24 h, 除掉目的蛋白中的咪唑。收集样品, 进行12% SDS-PAGE, 分析目的蛋白纯度。BCA试剂盒测定蛋白浓度, 计算产率。

1.2.4 Native-PAGE分析 取20 μg上述蛋白溶液进行Native-PAGE。Native-PAGE全程需在4 ℃条件下进行, 电泳缓冲液需要预冷, 蛋白样品不可在沸水浴中进行变性处理。配制电泳缓冲液和制胶时不可加入SDS, 5×Loading Buffer中不可加入SDS和β-巯基乙醇, 因为SDS和β-巯基乙醇均会导致蛋白质发生变性。

1.2.5 动态光散射实验(DLS) 将3种融合蛋白分别稀释为3.0 mg/mL, 然后取300 μL蛋白稀释液加入样品池, 使用马尔文粒径分析仪测量目的蛋白的粒径大小并分析其形成聚体的情况。

1.2.6 流式细胞术检测融合蛋白与凋亡细胞的结合能力 Jurkat淋巴瘤细胞用含10%胎牛血清的RPMI-1640, 37 ℃、5% CO_2条件下培养。每1×10^6个细胞用1 mL终浓度为25 μmol/L依托泊苷的培养液诱导6 h后, 在4 ℃条件下1 000 r/min离心5 min, 收集细胞, 每5×10^5个细胞用PBS缓冲液洗涤2次, 再用Binding Buffer缓冲液洗涤2次, 最终重悬于200 μL的Binding Buffer缓冲液中。将Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet融合蛋白提前定量稀释到一定浓度, 浓度分别为4、10、20、50、100、200、500、1 000、2 000、5 000 nmol/L, 分别取200 μL不同浓度融合蛋白的稀释液加入到200 μL细胞悬液中, 使融合蛋白的终浓度分别为2、5、10、25、50、100、250、500、1 000、2 500 nmol/L。冰上避光孵育30 min, 然后加入1 μL PI, 冰上避光孵育5 min。流式细胞仪检测: 根据融合蛋白的荧光光谱, 选择488 nm作为激发光波长, 用波长515 nm的通带滤器检测Citrine、Venus和Ypet(FL1), 用波长大于560 nm的滤器检测PI(FL-3)。

1.2.7 Citrine、Venus、Ypet三种荧光蛋白的氨基酸序列比对 根据NCBI中提供的Citrine、Venus、Ypet的蛋白质序列, 利用DNAMAN软件对这三种荧光蛋白进行多重序列比对。

1.2.8 统计学分析 所有实验数据以mean±S.D.表示, 统计学处理采用GraphPad Prim软件包, 组间差异采用单向方差分析(One-Way ANOVA), $P<0.05$为差异有统计学意义。

2 结果

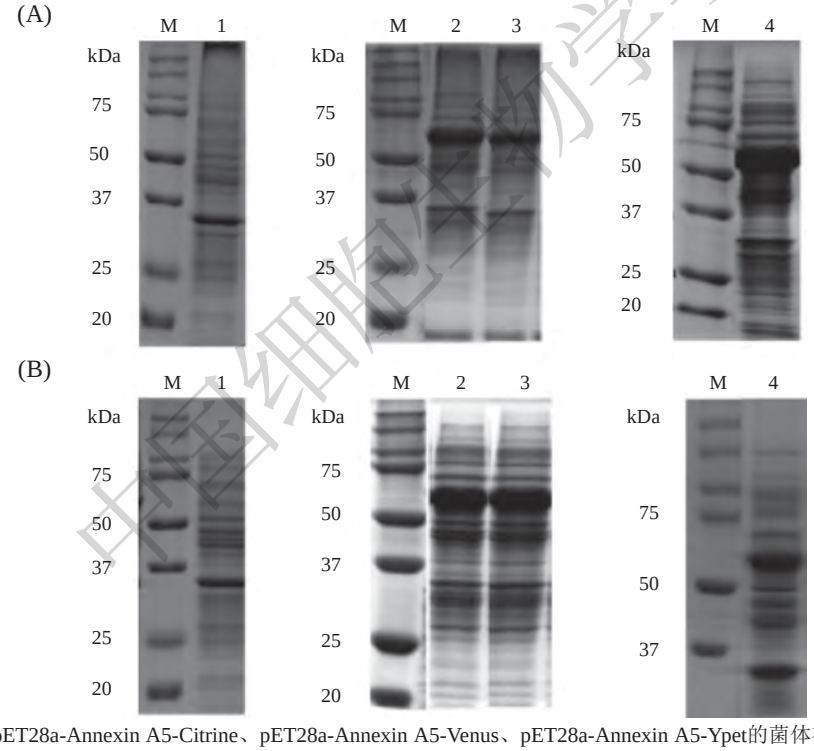
2.1 表达载体的构建

根据NCBI中*Citrine*、*Venus*和*Ypet*的cDNA序列设计特异性引物, 以本实验室保存的含有*Citrine*、

*Venus*和*Ypet*的重组质粒为模板, 通过PCR扩增出了*Citrine*、*Venus*和*Ypet*全长序列。经过*Nde* I和*Xho* I双酶切后, 将*Citrine*、*Venus*和*Ypet*片段插入到重组质粒pET28a-Annexin A5-EGFP-his的*Nde* I和*Xho* I位点之间, 取代原来EGFP的位置, 构建了C末端带有His-Tag的pET28a-Annexin A5-Citrine、pET28a-Annexin A5-Venus、pET28a-Annexin A5-Ypet表达质粒(图1)。菌落PCR鉴定及测序验证重组质粒中Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet的序列正确后, 转化大肠杆菌BL21(DE3)菌株。

2.2 Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet的小量表达

将重组质粒pET28a-Annexin A5-Citrine、pET28a-Annexin A5-Venus、pET28a-Annexin A5-Ypet转化至大肠杆菌BL21(DE3)后经IPTG诱导, 通过SDS-PAGE分析发现, 在37 ℃和20 ℃条件下诱导时, 在分子量约为65 kDa处均有目的蛋白表达条带(图2), 且与融合蛋白的理论分子量一致(表2), 而空载质粒在相应的分子量附近未出现明显的蛋白质条带(图2), 说明这三种融合蛋白在37 ℃和20 ℃条件下均成功表达。对37 ℃和20 ℃条件下目的蛋白的可溶性产量进行分析, 发现20 ℃诱导表达时目的蛋白在上清中的可溶性比例明显高于目的蛋白在37 ℃时的可溶性比例(图3)。结果表明: Annexin A5-Citrine在37 ℃和20 ℃条件下的可溶性比例分别为表达蛋白的68.59%和71.57%; Annexin A5-Venus在37 ℃和20 ℃条件下的可溶性比例分别为表达蛋白的73.44%和86.00%; 而Annexin A5-Ypet在37 ℃和20 ℃条件下的可溶性表达比例分别为表达蛋白的42.86%和85.42%。



A: 含pET28a vector、pET28a-Annexin A5-Citrine、pET28a-Annexin A5-Venus、pET28a-Annexin A5-Ypet的菌体在37 ℃诱导时的表达情况; M: protein marker; 1: 含pET28a vector的菌体总蛋白; 2: 含pET28a-Annexin A5-Citrine的菌体总蛋白; 3: 含pET28a-Annexin A5-Venus的菌体总蛋白; 4: 含pET28a-Annexin A5-Ypet的菌体总蛋白。B: 含pET28a vector, pET28a-Annexin A5-Citrine, pET28a-Annexin A5-Venus, pET28a-Annexin A5-Ypet的菌体在20 ℃诱导时的表达情况; M: protein marker; 1: 含pET28a vector的菌体总蛋白; 2: 含pET28a-Annexin A5-Citrine的菌体总蛋白; 3: 含pET28a-Annexin A5-Venus的菌体总蛋白; 4: 含pET28a-Annexin A5-Ypet的菌体总蛋白。

A: SDS-PAGE analysis of Annexin A5-Citrine, Annexin A5-Venus, Annexin A5-Ypet expression in the condition of 37 ℃; M: protein marker; 1: total crude protein of bacteria containing pET28a vector; 2: total crude protein of bacteria containing pET28a-Annexin A5-Citrine; 3: total crude protein of bacteria containing pET28a-Annexin A5-Venus; 4: total crude protein of bacteria containing pET28a-Annexin A5-Ypet. B: SDS-PAGE analysis of Annexin A5-Citrine, Annexin A5-Venus, Annexin A5-Ypet expression in the condition of 20 ℃; M: protein marker; 1: total crude protein of bacteria containing pET28a vector; 2: total crude protein of bacteria containing pET28a-Annexin A5-Citrine; 3: total crude protein of bacteria containing pET28a-Annexin A5-Venus; 4: total crude protein of bacteria containing pET28a-Annexin A5-Ypet.

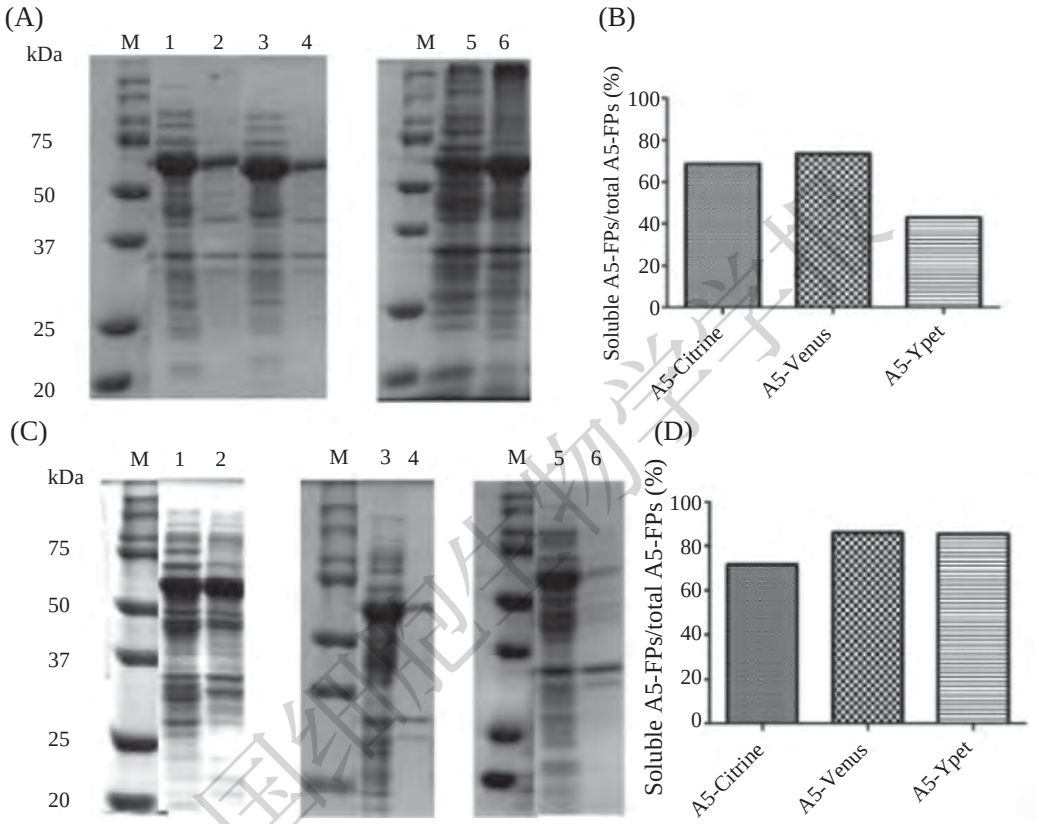
图2 Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet在37 °C和20 °C条件下表达的SDS-PAGE分析

Fig.2 SDS-PAGE analysis of Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet expression in the condition of 37 °C and 20 °C

表2 Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet的相对分子量

Table 2 The molecular weight of Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet

蛋白名称 Protein name	相对分子量(kDa) Molecular weight (kDa)
Annexin A5-Citrine	64.75
Annexin A5-Venus	64.60
Annexin A5-Ypet	64.57



A: 含pET28a-Annexin A5-Citrine、pET28a-Annexin A5-Venus、pET28a-Annexin A5-Ypet的菌体在37 ℃诱导时目的蛋白的可溶性表达情况; M: protein marker; 1、2: 含pET28a-Annexin A5-Citrine的菌体的上清和沉淀; 3、4: 含pET28a-Annexin A5-Venus的菌体的上清和沉淀; 5、6: 含pET28a-Annexin A5-Ypet的菌体的上清和沉淀。B: 可溶的Annexin A5-Citrine与总Annexin A5-Citrine、可溶的Annexin A5-Venus与总Annexin A5-Venus、可溶的Annexin A5-Ypet与总的Annexin A5-Ypet的比例; C: 含pET28a-Annexin A5-Citrine、pET28a-Annexin A5-Venus、pET28a-Annexin A5-Ypet的菌体在20 ℃诱导时目的蛋白的可溶性表达情况; M: protein marker; 1、2: 含pET28a-Annexin A5-Citrine的菌体的上清和沉淀; 3、4: 含pET28a-Annexin A5-Venus的菌体的上清和沉淀; 5、6: 含pET28a-Annexin A5-Ypet的菌体的上清和沉淀; D: 可溶的Annexin A5-Citrine与总Annexin A5-Citrine、可溶的Annexin A5-Venus与总Annexin A5-Venus、可溶的Annexin A5-Ypet与总的Annexin A5-Ypet的比例。

A: SDS-PAGE analysis of Annexin A5-Citrine, Annexin A5-Venus, Annexin A5-Ypet soluble expression in the condition of 37 ℃; M: protein marker; 1,2: supernatant protein and precipitate protein of bacteria containing pET28a-Annexin A5-Citrine, IPTG induction; 3,4: supernatant protein and precipitate protein of bacteria containing pET28a-Annexin A5-Venus, IPTG induction; 5,6: supernatant protein and precipitate protein of bacteria containing pET28a-Annexin A5-Ypet, IPTG induction; B: the ratio of soluble Annexin A5-Citrine to total Annexin A5-Citrine, soluble Annexin A5-Venus to total Annexin A5-Venus and soluble Annexin A5-Ypet to total Annexin A5-Ypet in 37 ℃ induction; C: SDS-PAGE analysis of Annexin A5-Citrine, Annexin A5-Venus, Annexin A5-Ypet soluble expression in the condition of 20 ℃; M: protein marker; 1,2: supernatant protein and precipitate protein of bacteria containing pET28a-Annexin A5-Citrine, IPTG induction; 3,4: supernatant protein and precipitate protein of bacteria containing pET28a-Annexin A5-Venus, IPTG induction; 5,6: supernatant protein and precipitate protein of bacteria containing pET28a-Annexin A5-Ypet, IPTG induction; D: the ratio of soluble Annexin A5-Citrine to total Annexin A5-Citrine, soluble Annexin A5-Venus to total Annexin A5-Venus and soluble Annexin A5-Ypet to total Annexin A5-Ypet in 20 ℃ induction.

图3 Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet在37 °C和20 °C条件下可溶性表达的SDS-PAGE分析

Fig.3 SDS-PAGE analysis of Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet soluble expression in the condition of 37 °C and 20 °C

2.3 Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet的表达和纯化

Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet融合蛋白表达后, 借助融合蛋白C末端的His-Tag, 利用Ni柱亲和层析纯化这3种融合蛋白, 纯化获得的蛋白浓度分别为10.37 mg/mL、4.85 mg/mL、5.37 mg/mL, 获得的蛋白质纯度均为90%左右(图4)。

2.4 Native-PAGE分析Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet融合蛋白形成聚体的情况

Annexin A5蛋白可以形成聚体, 荧光蛋白Citrine和Venus主要以单体形式存在, 而Ypet可以形成弱二聚体, 蛋白是否形成聚体会影响融合蛋白与磷脂酰丝氨酸的结合能力。为了探究Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet融合蛋白是否会形成聚体, 本文对这三种融合蛋白进行了Native-PAGE分析。结果表明, 这三种融合蛋白均只有一条明显的蛋白质条带, 说明没有聚体形成(图5)。

2.5 Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet融合蛋白的粒径分析

为了进一步探究Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet形成聚体的情况, 我们对这三种融合蛋白进行了动态光散射实验。结果表明这三种融合蛋白的粒径大小均一, 而且融合蛋白也不形成聚体(图6)。

2.6 Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet融合蛋白的凋亡检测分析

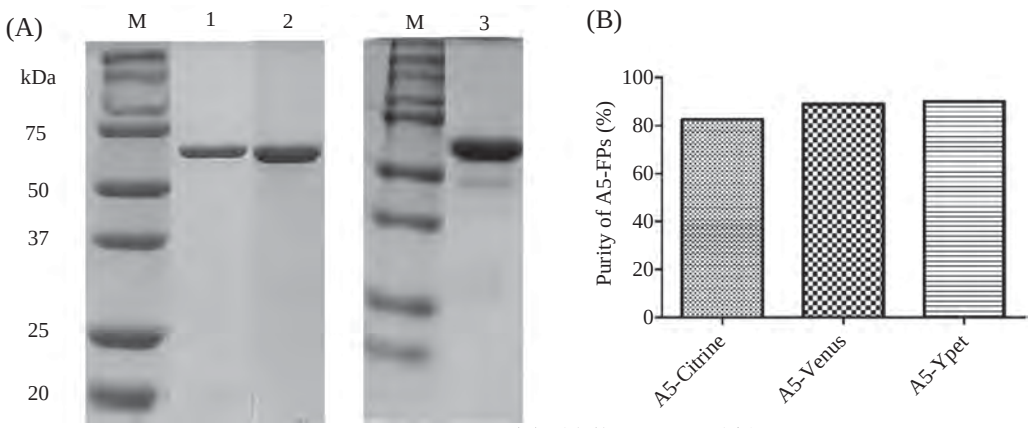
将纯化得到的Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet利用流式细胞术观察它们与凋亡细胞的结合情况。图7以Annexin A5-Citrine为例, 具体介绍利用流式细胞术评估凋亡检测功能的方法。设置不同的浓度梯度, 对细胞进行分群(图7A), 圈选Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet标记的凋亡细胞群(图7B)。对Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet标记的凋亡细胞表面的平均荧光强度进行分析(图8A~图8C)。结果表明, 三种融合蛋白的平均荧光强度均随着蛋白浓度的增加而增加, 说明纯化得到的Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet可与凋亡细胞发生特异性结合, 能够标记凋亡细胞。

同时, 我们对Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet与凋亡细胞的亲和力进行定量分析发现, Annexin A5-Citrine与凋亡细胞的亲和力为3 113.0 nmol/L(图8D), Annexin A5-Venus与凋亡细胞的亲和力为444.3 nmol/L(图8D), Annexin A5-Ypet与凋亡细胞的亲和力为391.6 nmol/L(图8D)。三者相比, Annexin A5-Venus和Annexin A5-Ypet与凋亡细胞的亲和力同属纳摩尔级, 且属于同一个数量级, 说明Annexin A5-Venus和Annexin A5-Ypet均可以用于细胞凋亡检测。而Annexin A5-Citrine与凋亡细胞的亲和力不如另外两个蛋白高, 虽然它也可以用于标记凋亡细胞, 但就效果而言, 却不如Annexin A5-Venus和Annexin A5-Ypet标记凋亡的效果好。

2.7 Citrine、Venus、Ypet三种荧光蛋白的氨基酸序列比对

以上结果显示, Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet三种融合蛋白在相对分子量、表达特性、形成聚体的情况、粒径分布等方面均没有明显差异, 但是它们与凋亡细胞结合时却表现出了截然不同的亲和力。于是, 我们对Citrine、Venus、Ypet的氨基酸序列进行比对分析以探究这三种荧光蛋白的结构差异对Annexin A5凋亡检测功能的影响。

Citrine、Venus、Ypet均由最初的黄色荧光蛋白EYFP改造而来, 通过氨基酸序列比对(图9), 我们发现了三者的氨基酸在多个位点上的差异(表3)。研究发现, F46L突变促进了发色团附近局部结构的改变, 因此促进了发色团的氧化, 这是发色团成熟的一个限速步骤[20]。此外, 也有研究发现, F64L突变造成了β-桶结构内部主要结构的变化, 从而阻止了卤化物离子进入到发色团附近的结合腔[20]。M153T、V163A、S175G这三个重要的突变在β-桶外的loop区引入了高度灵活的、更小的侧链, 从而促进了荧光蛋白的成熟过程。F46L、F64L、M153T、V163A、S175G是Venus和Ypet区别于最初的EYFP最重要的五个突变, 而Citrine在这五个位点的氨基酸并没有改变, 这也许可以解释为什么Annexin A5-Citrine与凋亡细胞的亲和力比Annexin A5-Venus、Annexin A5-Ypet与凋亡细胞的亲和力小, 说明黄色荧光蛋白的结构会影响Annexin A5与凋亡细胞的结合。与Annexin A5-Venus相比, Annexin A5-Ypet在

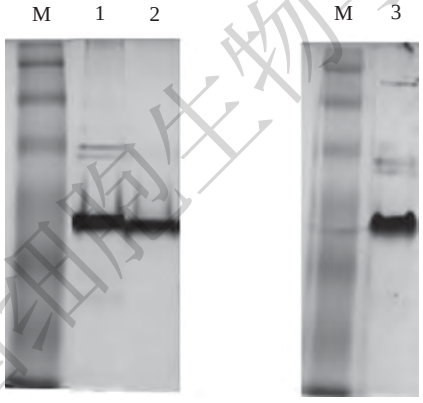


A: 纯化后的Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet融合蛋白的SDS-PAGE分析; M: protein marker; 1: Annexin A5-Venus; 2: Annexin A5-Ypet; 3: Annexin A5-Citrine。B: 通过Ni柱亲和层析纯化的Annexin A5-Citrine、Annexin A5-Venus和Annexin A5-Ypet融合蛋白纯度分析。

A: SDS-PAGE analysis of purified Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet; M: protein marker; 1: Annexin A5-Venus; 2: Annexin A5-Ypet; 3: Annexin A5-Citrine. B: the purity analysis of Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet fusion protein purified by Ni column affinity chromatography.

图4 通过Ni柱亲和层析纯化的Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet融合蛋白的SDS-PAGE分析

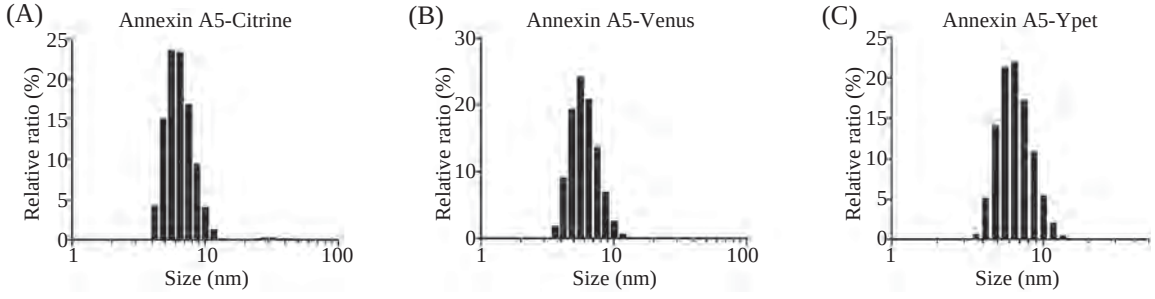
Fig.4 SDS-PAGE analysis of Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet purified by Ni column affinity chromatography



M: protein marker; 1: Annexin A5-Citrine; 2: Annexin A5-Venus; 3: Annexin A5-Ypet.

图5 通过Ni柱亲和层析纯化的Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet融合蛋白的Native-PAGE分析

Fig.5 Native-PAGE analysis of Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet purified by Ni column affinity chromatography

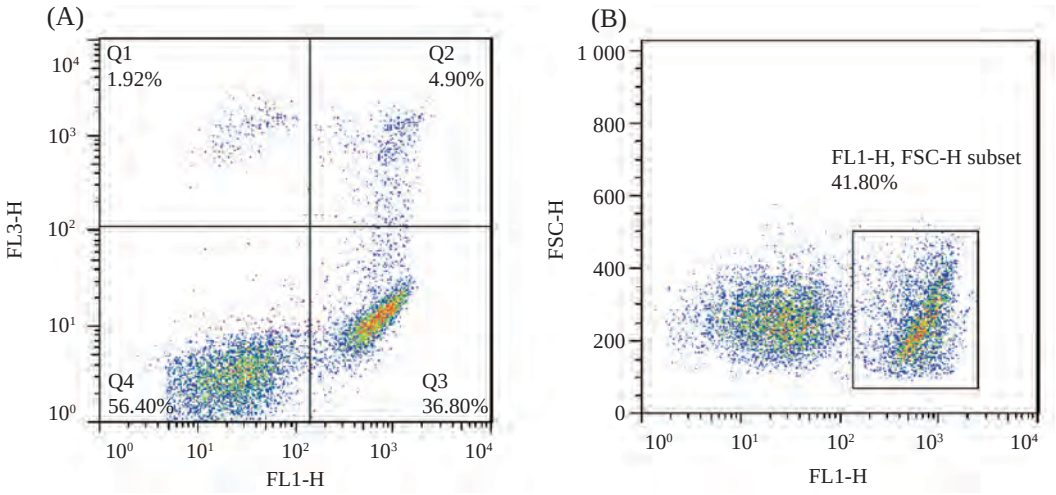


A: Annexin A5-Citrine的粒径分布图; B: Annexin A5-Venus的粒径分布图; C: Annexin A5-Ypet的粒径分布图。

A: the particle size distribution of Annexin A5-Citrine; B: the particle size distribution of Annexin A5-Venus; C: the particle size distribution of Annexin A5-Ypet.

图6 Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet融合蛋白的粒径分布图

Fig.6 The particle size distribution of Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet fusion protein

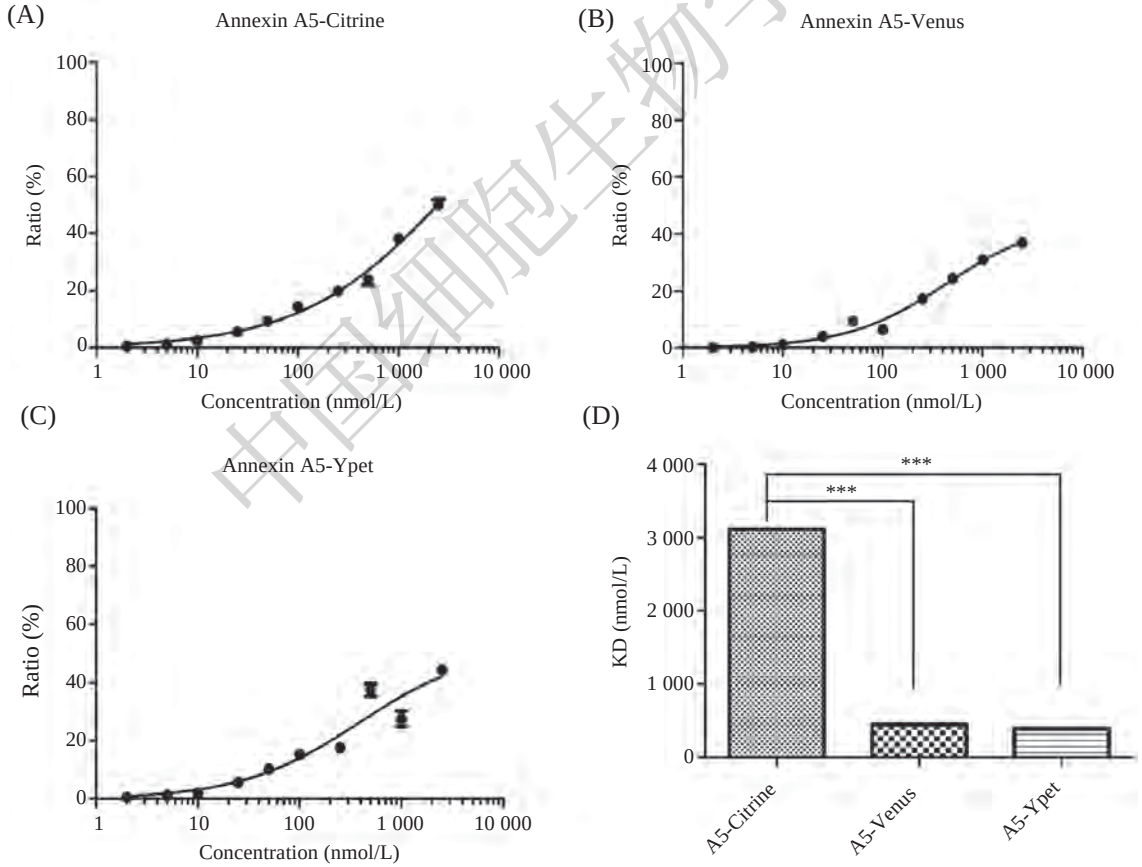


A: Annexin A5-Citrine标记凋亡细胞的散点图; B: 利用流式细胞术定量凋亡细胞比例的散点图。

A: diagrammatic dot-plot of apoptotic cells labeled with Annexin A5-Citrine; B: diagrammatic dot-plot of flow cytometric measurement of apoptotic cells.

图7 利用流式细胞术评估Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet的凋亡检测功能

Fig.7 Evaluation of apoptotic detection ability of Annexin-Citrine, Annexin-Venus, Annexin A5-Ypet by flow cytometry



A~C: Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet与凋亡细胞结合的平均荧光强度值分析。D: Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet与凋亡细胞的亲和力, ***P<0.001。

A-C: analysis of median fluorescence intensity of Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet binding to apoptotic cells; D: the affinity of Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet with apoptotic cells, ***P<0.001.

图8 Annexin A5-Citrine, Annexin A5-Venus和Annexin A5-Ypet的凋亡检测功能评估

Fig.8 Evaluation of apoptotic detection ability of Annexin A5-Citrine, Annexin A5-Venus and Annexin A5-Ypet

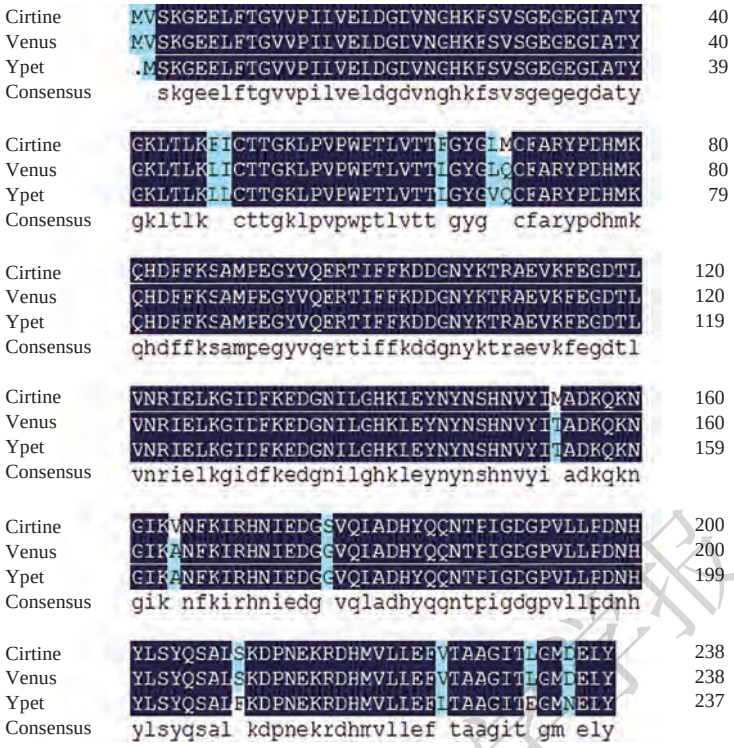


图9 Citrine、Venus和Ypet的氨基酸序列比对

Fig.9 Alignment of amino acid sequences of Citrine, Venus and Ypet

表3 Citrine、Venus、Ypet的氨基酸序列差异

Tabel 3 Comparison of sequence difference between Citrine, Venus and Ypet

名称 Name	氨基酸残基 Amino acid residue											亲和力(nmol/L) Affinity (nmol/L)
	46	47	64	68	69	153	163	175	208	224	231	234	
Citrine	Phe	ILe	Phe	Leu	Met	Met	Val	Ser	Ser	Val	Leu	Asp	3 113.0
Venus	Leu	ILe	Leu	Leu	Gln	Thr	Ala	Gly	Ser	Val	Leu	Asp	444.3
Ypet	Leu	Leu	Leu	Val	Gln	Thr	Ala	Gly	Phe	Leu	Glu	Asn	391.6

208、224、231、234位的差异可能有造成两者亲和力有所差异的结构原因。

3 讨论

Annexin A5是膜联蛋白家族的成员之一, 它能够钙依赖性地与凋亡细胞表面的磷脂酰丝氨酸结合, 因而可以用于凋亡检测。自从被开发为凋亡探针至今, Annexin A5被广泛用于体外凋亡检测。目前, 化学偶联方法标记的Annexin A5被广泛使用, 如FITC(异硫氰酸荧光素)标记的Annexin A5。该探针的制备过程非常复杂繁琐, 而且氨基介导的化学修饰过程还会导致Annexin A5与凋亡细胞PS的结合能力下降。另外, 化学标记Annexin A5的最终产物通常是不同标记程度的混合物, 非常不均一。研究发现, 融合了荧光蛋白的Annexin A5相对均一, 且荧光更强、更亮、更稳定[16-17,19-20]。目前市场上只有Annexin A5-EGFP绿色的荧光蛋白融合分子被广泛应用于细胞凋亡检测。

黄色荧光蛋白作为绿色荧光蛋白的一种变体, 其荧光向红色光谱偏移, 最大激发波长为514 nm, 最大发射波长为527 nm, 因此具有比绿色荧光更为广泛的应用面。这主要是由于蛋白203位苏氨酸变为酪氨酸。最初被使用的黄色荧光蛋白是EYFP, 目前有三种改良的黄色荧光蛋白: Citrine、Venus、Ypet, 三者的氨基酸序列相似性达98%以上。我们选择这三种黄色荧光蛋白与Annexin A5融合制备凋亡检测探针, 结果表明, Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet与凋亡细胞的结合能力差

异很大, 其亲和力大小是Annexin A5-Ypet>Annexin A5-Venus>Annexin A5-Citrine。这三种融合蛋白在相对分子量、表达特性、形成聚体的情况、粒径分布等方面均没有明显差异, 于是我们猜测荧光蛋白的氨基酸序列可能影响了Annexin A5与凋亡细胞的结合。我们通过查阅文献发现, 与EYFP相比, 改良的黄色荧光蛋白有五个重要的突变, 分别是*F46L*、*F64L*、*M153T*、*V163A*、*S175G*, 这些突变对荧光蛋白的特性有重要的影响。比对结果表明, Venus和Ypet含有这五个位点的突变, 而Citrine不含有这五个位点的突变, 这也许是Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet与凋亡细胞结合能力差异很大的原因。此外, 我们还发现, Annexin A5-Venus与Annexin A5-Ypet在S208P、V224L、L231E、D234N位的差异可能有造成两者亲和力有所差异的结构原因。以上结果说明, 不同黄色荧光蛋白在结构上的细微差异仍然能够较显著地影响Annexin A5与凋亡细胞的结合。

本文成功实现了Annexin A5-Citrine、Annexin A5-Venus、Annexin A5-Ypet在原核系统中的可溶性高效表达, 提供了大量的高纯度融合蛋白, 研究了这三种蛋白与凋亡细胞表面PS的结合能力, 并分析了荧光蛋白在氨基酸组成上的细微差异对Annexin A5与凋亡细胞结合的影响。这为进一步研究荧光蛋白标记的Annexin A5探针的功能和潜在应用奠定了基础。

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