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· 论著 ·

柔嫩艾美耳球虫MIC1与MIC2相互作用的鉴定*

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【摘要】 **目的** 观察柔嫩艾美耳球虫基因微线蛋白1(EtMIC1)和微线蛋白2(EtMIC2)之间的相互作用,为研究微线蛋白之间相互影响的分子机制以及该互作在鸡球虫入侵宿主细胞过程中的作用提供参考依据。 **方法** 采用PCR方法克隆柔嫩艾美耳球虫EtMIC1和EtMIC2基因,构建不同的重组表达载体。将构建的酵母双杂交诱饵质粒pGBKT7-EtMIC1及捕获质粒pGADT7-EtMIC2转化至Y2HGold酵母感受态,涂布于对应的营养缺陷培养基进行毒性和自激活活性检测。表达GST-EtMIC1和His-EtMIC2两种融合蛋白,纯化后通过GST-Pull down验证二者在体外的相互作用。将构建的pcDNA3.1-His-EtMIC1和pcDNA3.1- HA-EtMIC2重组质粒转染HEK-293T细胞,RIPA裂解后取上清进行免疫共沉淀试验,鉴定二者在体内的相互作用。将真核表达载体pEtMIC1-Myc-LC151和pEtMIC2-HA-KN151共转染HEK-293T细胞,激光共聚焦显微镜观察转染后的细胞内发出的红色荧光。 **结果** pGBKT7-EtMIC1和pGADT7-EtMIC2单转化对酵母细胞无明显毒性作用,且自身无自激活活性,而pGBKT7-EtMIC1/ pGADT7-EtMIC2共转化后能够在显色板上长出蓝色菌落,说明二者在细胞内可相互作用;GST-Pull down试验表明二者可在体外发生相互作用;免疫共沉淀和双分子荧光互补试验证实EtMIC1和EtMIC2在细胞内相互作用。 **结论** EtMIC1和EtMIC2在体内、外均能够产生相互作用,为进一步探索微线蛋白之间相互影响的分子机制以及该互作在鸡球虫入侵宿主细胞过程中的作用提供参考依据。

【关键词】 柔嫩艾美尔球虫;微线蛋白1;微线蛋白2;相互作用

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Identification of interaction between *Eimeria tenella* MIC1 and MIC2

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【Abstract】 **Objective** The molecular mechanism of interaction between *Eimeria tenella* MIC1(EtMIC1) and MIC2 (EtMIC2) were investigated, thus providing a reference for the invasion of chiken coccidian into host cells. **Methods** The EtMIC1 and EtMIC2 genes were cloned by PCR using the cDNA of *Eimeria tenella* sporozoite as templates. The yeast two-hybrid bait plasmid pGBKT7-EtMIC1 and prey plasmid pGADT7-EtMIC2 were transformed into Y2HGold, cultured on the auxotrophic selection medium for investigating the toxicity and self-activation. For GST-Pull down assay, the GST-EtMIC1 and His-EtMIC2 fusion proteins were expressed and purified. The recombinant plasmids pcDNA3.1-His-EtMIC1 and pcDNA3.1-HA-EtMIC2 were transfected into HEK-293T cells to certify the interaction in vivo with co-immunoprecipitation using the RIPA lysate supernatant. The pEtMIC1-Myc-LC151 and pEtMIC2-HA-KN151 were co-transfected into HEK-293T cells for the observation of red fluorescence by confocal laser scanning microscopy. **Results** EtMIC1 and EtMIC2 were not toxic and self-activating to yeast cells, while the co-transformation of pGBKT7-ETMIC1/ PGADT7-ETMIC2 could activate the yeast two-hybrid reporting system, which showed that EtMIC1 and EtMIC2 could interact in vivo. While the GST-Pull down results showed that EtMIC1 and EtMIC2 could interact in vitro. The interaction between EtMIC1 and EtMIC2 in 293T cells was furtherly confirmed by co-immunoprecipitation and bimolecular fluorescence complementation assays. **Conclusion** EtMIC1 and EtMIC2 could interact each other in vitro and in 293T cells, which provided a reference for exploring the molecular mechanism of interaction and the invasion of chiken coccidian into host cells.

【Key words】 *Eimera tenella*; microneme protein1; microneme protein2; interaction

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鸡球虫病是一种由艾美耳属球虫引起以肠道出血、腹泻为主要特征的胞内寄生原虫病，其中柔嫩艾美耳球虫(*Eimera tenella*)是危害最严重的的病原性寄生虫之一[1-2]。目前球虫病的防治以化学药物和疫苗预防为主要手段，但对于球虫与宿主细胞的关系尤其是入侵宿主细胞的分子机制尚不明确，因此仍无有效办法控制鸡球虫病的发生[3-4]。球虫子孢子入侵宿主细胞是感染机体的第一步，有研究表明顶器中微线体、棒状体和致密颗粒等共同参与该入侵过程，且微线体蛋白在其中起关键作用[5-6]。微线蛋白1(microneme protein1,MIC1)是最早报道的微线蛋白，由712个氨基酸构成，是血小板反应相关蛋白家族成员且具有该家族的保守结构区域，通常在球虫子孢子和裂殖子无性繁殖阶段表达[7]。微线蛋白2(microneme protein2,MIC2)是一种微线体分泌的酸性蛋白，在子孢子入侵动物机体后会迅速分泌并覆盖于孢子生殖和裂殖生殖阶段的虫体以及整个宿主细胞表面[8]，由此推测EtMIC2蛋白在球虫入侵宿主细胞过程中发挥重要作用。目前对于顶复门原虫MIC1与MIC2相互作用的研究较少，尚未见关于柔嫩艾美尔球虫MIC1和MIC2之间存在相互作用的报道。本研究利用GST-Pull down技术、免疫共沉淀以及双分子荧光互补等方法验证柔嫩艾美耳球虫MIC1与MIC2之间的相互作用，为进一步探索微线蛋白之间相互影响的分子机制以及该互作在鸡球虫入侵宿主细胞过程中的作用提供参考依据。

材料与方法

1 材料

1.1 虫株、载体 柔嫩艾美耳球虫孢子化卵囊，HEK-293T细胞，pET-32a(+)、pGEX-4T-、pcDNA3.1-His、pcDNA3.1-HA、pbJUN-HA-KN151、pbFOS-Myc-LC151、pMyc-LC151及pHA-KN151载体均由吉林大学动物医学学院寄生虫实验室保存。

1.2 主要试剂 限制性内切酶BamHI、NdeI、SalI、XhoI、XbaI、NheI、PvuI及T4DNA连接酶为日本Takara公司产品；鼠源Anti-GST抗体、Anti-His抗体，Anti-HA抗体及山羊抗鼠HRP-IgG抗体为美国Proteintech公司产品；Lipofectamine 2000为美国Invitrogen公司产品；蛋白A磁珠为美国Millipore公司产品；无内毒素质粒DNA小量提取试剂盒购于上海生工公司；His标签蛋白纯化试剂盒和GST琼脂糖凝胶购于北京康为世纪公司。

2 方法

2.1 表达载体的构建 根据GenBank登录的EtMIC1(EU093966)和EtMIC2(AF111839)基因序列，采用Oligo7软件设计8对特异性引物(表1)，以柔嫩艾美耳球虫cDNA为模板克隆EtMIC1和EtMIC2基因，分别构建pGBKT7-EtMIC1/pGADT7-EtMIC2、pGEX-4T-EtMIC1/pET-32a-EtMIC2、pcDNA3.1-His-EtMIC1/pcDNA3.1-HA-EtMIC2、pEtMIC1-Myc-LC151/pEtMIC2-HA-KN151重组质粒，用表1中对应的限制性内切酶双酶切鉴定并测序。

2.2 毒性和自激活作用检测 将pGBKT7-EtMIC1和pGADT7-EtMIC2分别转化Y2HGold酵母感受态，并将部分转化液均匀涂布于对应的不同营养缺陷培养基上，观察菌落生长状态。将pGBKT7-EtMIC1和pGADT7-EtMIC2共转化至Y2HGold感受态，分别涂布于TDO/X和QDO/X/A显色板上，观察菌落生长状态和颜色变化。如果试验组和阳性对照组的酵

表1 引物信息及PCR反应条件(下划线处为酶切位点)

Table 1 List of primer sequences and PCR reaction conditions (underlined the sequence is the restriction site)

引物名称	引物序列(5'→3')	PCR反应条件	酶切位点
pGBKT7-EtMIC1-F	CATATGGGCGCAACTACCAGCTCT	95 ℃ 5 min;30 cycles:95 ℃ 30 s,63 ℃ 30 s,72 ℃ 150 s,72 ℃ 10 min	NdeI/BamHI
pGBKT7-EtMIC1-R	GGATCCTGCCCACATCTCTGATTGTT		
pGADT7-EtMIC2-F	CATATGATGGTTAGGACGAGAGTCCCAGG	95 ℃5 min;30 cycles:95 ℃30 s,62 ℃30 s,72 ℃60 s,72 ℃10 min	NdeI/BamHI
pGADT7-EtMIC2-R	GGATCCTCAGGATGACTGTTGAGTGTCA		
pGEX-4T-EtMIC1-F	GGATCCGGCGCAACTACCAGCTCT	95 ℃5 min;30 cycles:95 ℃30 s,60 ℃30 s,72 ℃150 s,72 ℃10 min	BamHI/Sal I
pGEX-4T-EtMIC1-R	GTCGACTGCCCACATCTCTGATTGTT		
pET-32a-EtMIC2-F	GGATCCGTTAGGACGAGAGTCCCAG	95 ℃5 min;30 cycles:95 ℃30 s,62 ℃30 s,72 ℃60 s,72 ℃10 min	BamHI/XhoI
pET-32a-EtMIC2-R	CTCGAGTCAGGATGACTGTTGAGTGTCA		
pcDNA3.1-His-EtMIC1-F	GGATCC ACCATGGGCGCAACTACCAGCTCT	95 ℃5 min;30 cycles:95 ℃30 s,62 ℃30 s,72 ℃150 s,72 ℃10 min	BamHI/XbaI
pcDNA3.1-His-EtMIC1-R	TCTAGA GATGCCCACATCTCTGATTGTT		
pcDNA3.1-HA-EtMIC2-F	GCTAGCCACCATGGTTAGGACGAGAGTCCCAG	95 ℃5 min;30 cycles:95 ℃30 s,62 ℃30 s,72 ℃60 s,72 ℃10 min	NheI/XhoI
pcDNA3.1-HA-EtMIC2-R	CTCGAGCTGTTGAGTGTCACTCTCTGCAGC		
pEtMIC1-Myc-LC151-F	GCTAGCCACCATGGGCGCAACTACCAGCTCT	95 ℃5 min;30 cycles:95 ℃30 s,62 ℃30 s,72 ℃150 s,72 ℃10 min	NheI/PvuI
pEtMIC1-Myc-LC151-R	CGATCG ATGCCCACATCTCTGATTGTT		
pEtMIC2-HA-KN151-F	GCTAGCCACCATGGTTAGGACGAGAGTCCCAG	95 ℃5 min;30 cycles:95 ℃30 s,62 ℃30 s,72 ℃60 s,72 ℃10 min	NheI/XhoI
pEtMIC2-HA-KN151-R	CTCGAGCCCTGTTGAGTGTCACTCTCTGCAGC		

母菌落均呈蓝色,而阴性对照组既不生长也不显色,则初步判定二者可产生相互作用。

2.3 GST-Pull down 验证 使用自诱导培养基ZYM-5052原核诱导表达pGEX-4T-EtMIC1和pET-32a-EtMIC2重组蛋白后离心收集菌体沉淀并超声破碎,对上清中的可溶性蛋白GST-EtMIC1、His-EtMIC2分别用GST琼脂糖凝胶和His标签蛋白纯化试剂盒纯化后进行GST-Pull down验证。

2.4 免疫共沉淀验证 将稀释的重组质粒和脂质体2000转染至HEK-293T细胞。收集细胞,用RIPA裂解后取部分上清分别用Anti-His和Anti-HA抗体4℃孵育过夜,再用预处理的蛋白A磁珠和孵育过的鼠源抗体继续4℃孵育,细胞裂解液处理后收集洗脱液进行Western blot分析。

2.5 双分子荧光互补验证 将测序正确的真核表达载体pEtMIC1-Myc-LC151和pEtMIC2-HA-KN151转染至HEK-293 T细胞,培养4~6 h后更换培养基继续培养20 h,弃掉培养基,用PBS洗涤2~3次,4%多聚甲醛室温固定10 min,300 μl DAPI室温避光染色8 min,最后滴加适量抗荧光衰减封片剂进行封片,用激光共聚焦显微镜观察细胞内出现的红色荧光。

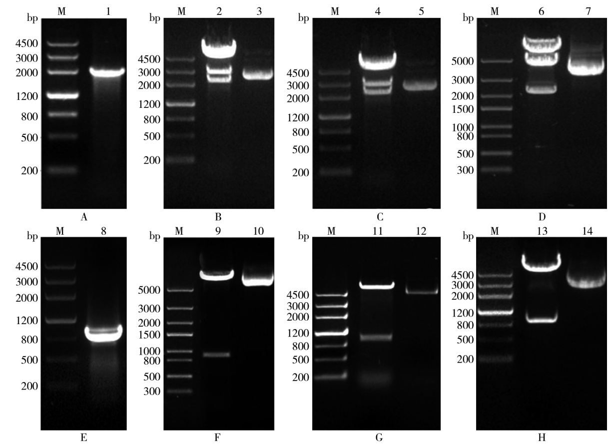
结 果

1 重组质粒的构建及鉴定

PCR扩增产物EtMIC1和EtMIC2经10 g/L琼脂糖凝胶电泳分析,大小分别为2 025 bp和969 bp,与预期相符(图1A、B)。以上述基因片段构建酵母双杂交重组质粒pGBKT7-EtMIC1和pGADT7-EtMIC2,GST-Pull down表达载体pGEX-4T-EtMIC1和pET-32a-EtMIC2,免疫共沉淀表达载体pcDNA3.1-His-EtMIC1和pcDNA3.1-HA-EtMIC2,经双酶切和测序鉴定均构建正确(图1C-H)。

2 毒性和自激活活性

pGBKT7-EtMIC1和pGADT7-EtMIC2单转化组的酵母菌落与pGBKT7-p53/ pGADT7-T阳性对照组菌落大小无明显差异,说明EtMIC1和EtMIC2对酵母细胞无明显毒性作用(图2A、B)。单转化重组质粒不能在TDO/X及QDO/X/A平板上生长,证明质粒自身无自激活活性(图2A、B)。将含有pGBKT7-EtMIC1和pGADT7-EtMIC2重组质粒的酵母细胞进行α-半乳糖苷酶试验,结果显示共转化组与阳性对照组菌落显蓝色,而阴性对照组(pGBKT7-lam/pGADT7-T)无显色现象,初步证实EtMIC1与EtMIC2在真核细胞中有相互作用(图2C、D、E)。



A EtMIC1 PCR产物 B pGBKT7-EtMIC1双酶切产物 C pGEX-4T-EtMIC1双酶切产物 D pcDNA3.1-His-EtMIC1双酶切产物 E EtMIC2 PCR产物 F pGADT7-EtMIC2双酶切产物 G pET-32a-EtMIC2双酶切产物 H pcDNA3.1-HA-EtMIC2双酶切产物

图1 重组质粒双酶切鉴定

A EtMIC1 PCR Amplification product B Double enzyme digestion of pGBKT7-EtMIC1 C Double enzyme digestion of pGEX-4T-EtMIC1 D Double enzyme digestion of pcDNA3.1-His-EtMIC1 E EtMIC2 PCR Amplification product F Double enzyme digestion of pGADT7-EtMIC2 G Double enzyme digestion of pET-32a-EtMIC2 H Double enzyme digestion of pcDNA3.1-HA-EtMIC2

Fig.1 Identification of the recombinant plasmids by double enzymatic digestion

3 GST-Pull down验证

取GST-Pull down洗脱液进行Western blot,结果如图3。用Anti-His抗体孵育的GST-EtMIC1/His-EtMIC2洗脱液中检测到His-EtMIC2融合蛋白,GST标签蛋白/His-EtMIC2共孵育液中未检测到His-EtMIC2融合蛋白;用Anti-GST抗体孵育的GST-EtMIC1/ His-EtMIC2洗脱液中检测到GST-EtMIC1融合蛋白,GST-pGEX-4T-1/His-EtMIC2共孵育液中也检测到GST标签蛋白(图3),表明EtMIC1与EtMIC2在体外发生了相互作用。

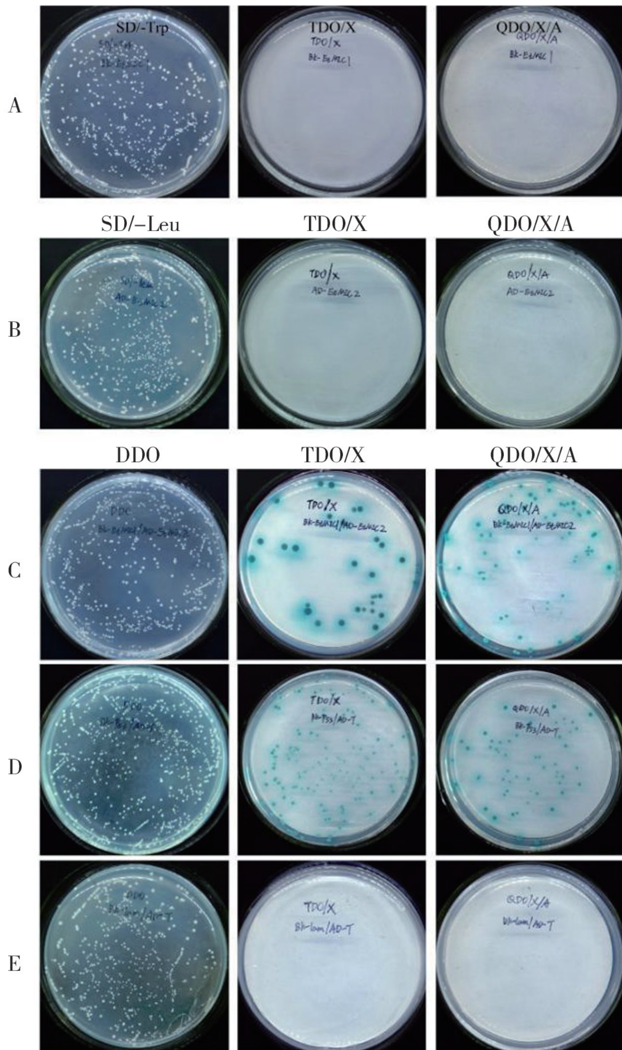
4 免疫共沉淀验证

Western blot显示pcDNA3.1-His-EtMIC1/pcDNA3.1-HA-EtMIC2共转染组与蛋白A磁珠、Anti-His抗体孵育后的复合物中检测到His-EtMIC1和HA-EtMIC2融合蛋白,大小分别约为75 ku和34 ku。单转染组只检测到His-EtMIC1,而未检测到HA-EtMIC2,表明His-EtMIC1和HA-EtMIC2在真核细胞内产生了相互作用。用Anti-HA抗体孵育后也可证明二者在体内存在相互作用(图4)。

5 双分子荧光互补验证

经激光共聚焦显微镜观察,pEtMIC1-Myc-LC151/pEtMIC2-HA-KN151共转染组和pbFOS-Myc-LC151/pbJUN-HA-KN151阳性对照组HEK-293T细胞内均发出明亮的红色荧光,而阴性对照组无

此红色荧光(图5),表明 EtMIC1 与 EtMIC2 在真核细胞中存在相互作用。

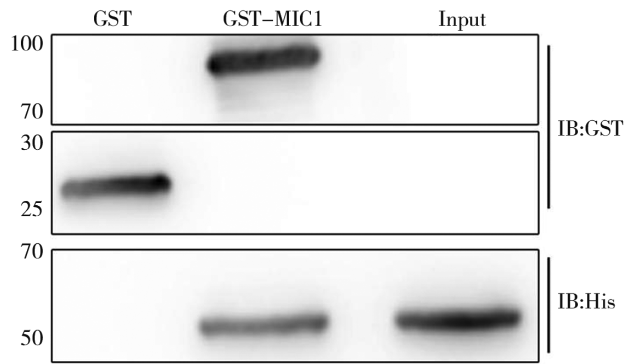


A pGBKT7-EtMIC1 单转化组 B pGADT7-EtMIC2 单转化组 C pGBKT7-EtMIC1/pGADT7-EtMIC2 共转化组 D pGBKT7-p53/pGADT7-T 阳性对照组 E pGBKT7-lam/pGADT7-T 阴性对照组

图2 酵母双杂交验证 EtMIC1 与 EtMIC2 的相互作用

A pGBKT7-EtMIC1 single transformation group B pGADT7-EtMIC2 single transformation group C pGBKT7-EtMIC1/pGADT7-EtMIC2 co-transformation group D Positive control: pGBKT7-p53/pGADT7-T co-transformation group E Negative control: pGBKT7-lam/pGADT7-T

Fig. 2 Identification of the interaction of EtMIC1 and EtMIC2 by yeast two-hybridization



GST GST 标签蛋白与 His-EtMIC2 共孵育组 GST-EtMIC1 GST-EtMIC1 与 His-EtMIC2 共孵育组 Input His-EtMIC2 蛋白对照组

图3 GST-Pull down 验证 EtMIC1 与 EtMIC2 的相互作用

GST Incubation of GST and His-EtMIC2 GST-EtMIC1 Incubation of GST-EtMIC1 and His-EtMIC2 Input His-EtMIC2

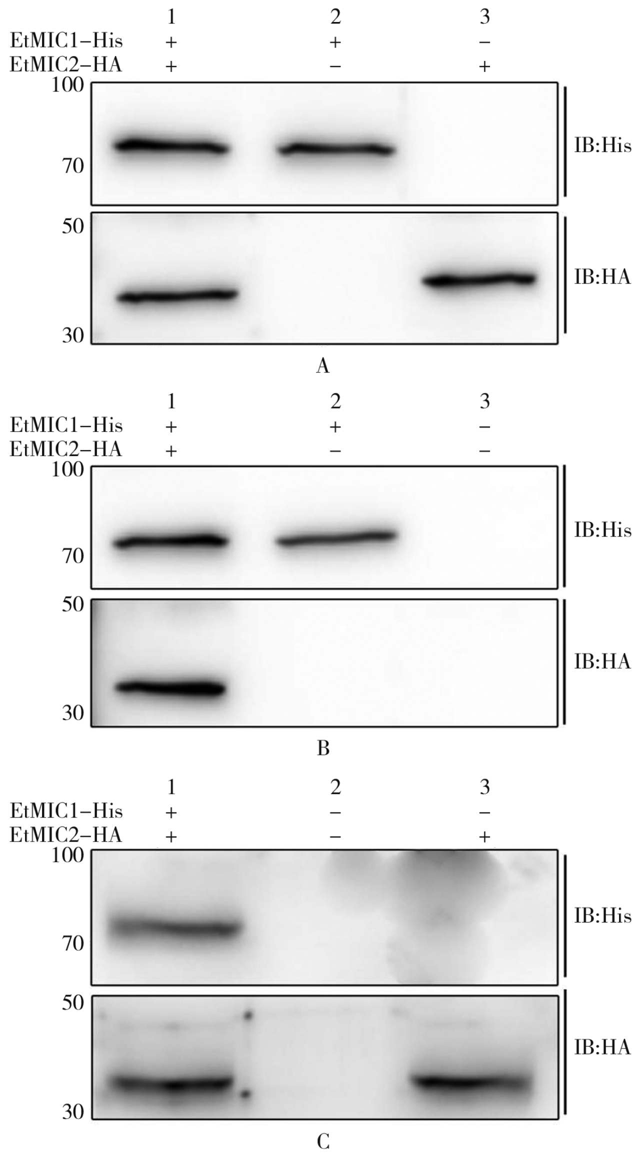
Fig. 3 Identification of the interaction of EtMIC1 and EtMIC2 by GST-Pull down

讨论

柔嫩艾美耳球虫属于顶复门原虫,在虫体的顶部有一特殊结构顶复合器,其中含有微线体、棒状体、致密颗粒等一系列分泌器官,而最重要的是位于子孢子和裂殖子顶端的微线体,其数量在不同种属寄生虫以及寄生虫的不同发育阶段均不相同[9]。目前弓形虫、柔嫩艾美耳球虫等虫体的微线体蛋白可通过蛋白质组学、梯度纯化其顶部分泌器官的方法进行鉴定[10-11]。顶复门原虫通过微线蛋白之间或微线蛋白与棒状体蛋白之间的共同作用引导虫体主动入侵宿主细胞[12]。目前已有多种方法用于研究蛋白质之间的相互作用,例如酵母双杂交技术、GST-Pull down 试验、免疫共沉淀技术、双分子荧光互补试验等。Scholtyseck 等[13]利用酵母双杂交技术从柔嫩艾美尔球虫子孢子 cDNA 文库中筛选到 EtMIC2 与 EtAMA1 有相互作用。赵娜[14]构建了柔嫩艾美尔球虫酵母双杂交 cDNA 文库并筛选到与端粒酶逆转录酶(TERT)能够产生相互作用的14-3-3蛋白,同时用 GST-Pull down、免疫共沉淀技术进一步证实了两种蛋白的相互作用。本研究利用双分子荧光互补、免疫共沉淀等技术验证柔嫩艾美尔球虫 MIC1 蛋白与 MIC2 蛋白有相互作用。

在酵母双杂交系统中,如果外源蛋白对酵母菌生长有毒性会导致菌落生长缓慢,进而影响结果的正确判定。研究表明有些外源蛋白在酵母细胞中能够单独启动下游报告基因转录,从而产生自激活现象,所以检测外源蛋白对酵母细胞的毒性和自激活活性非常重要[15]。本研究中 pGBKT7-EtMIC1 和 pGADT7-EtMIC2 在相应营养缺陷培养基生长的酵母菌落大小无明显差异,表明二者对酵母生长不产生明显的毒性作用。α-半乳糖苷酶试验结果表明 EtMIC1 与 EtMIC2 自身均不具有自激活活性,而 pGBKT7-EtMIC1 和 pGADT7-EtMIC2 共转化的酵母菌在显色板上能够生长出蓝色菌落,说明二者在酵母细胞内产生了相互作用。在进行 GST-Pull down 试验时,为保证融合蛋白的正确折叠和生物学活性,本研究采用自诱导培养基 ZYM-5052 进行 GST-EtMIC1 和 His-EtMIC2 原核蛋白的诱导和表达[16]。考虑到单独的 GST 标签蛋白与 His-EtMIC2 融合蛋白不能产生相互作用,因此在试验中用 GST 标签蛋白作阴性对照,结果表明 EtMIC1

与EtMIC2在体外存在互作。由于GST-Pull down试验也可能存在假阳性结果,因此采用免疫共沉淀技术进一步验证EtMIC1与EtMIC2在体内的相互作用。结果显示,在Anti-His抗体沉淀下来的复合物中检测到HA-EtMIC2,同样在Anti-HA抗体沉淀复合物中也检测到His-EtMIC1,证明了HA-EtMIC2和His-EtMIC1在真核细胞内发生相互作用。对EtMIC1与

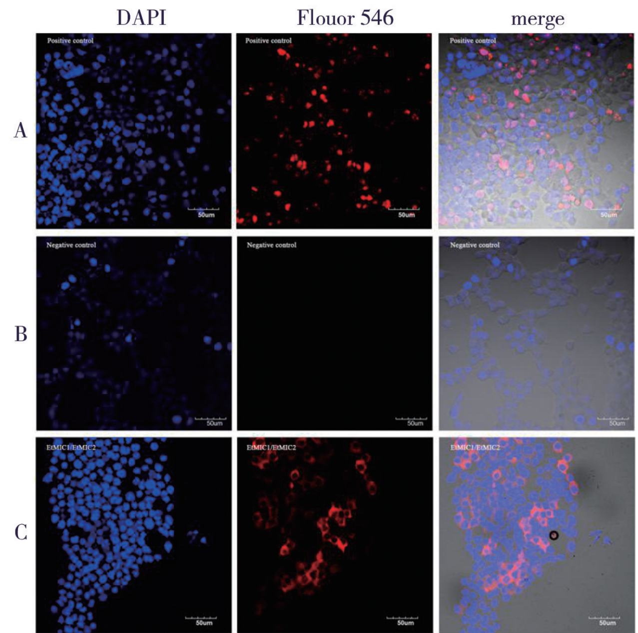


A,B,C 分别为细胞裂解液,Anti-His免疫沉淀复合物和Anti-HA免疫沉淀复合物 1 pcDNA3.1-His-EtMIC1/ pcDNA3.1-HA-EtMIC2共转染组 2 pcDNA3.1-His-EtMIC1单转染组 3 pcDNA3.1-HA-EtMIC2单转染组 IB:His Anti-His抗体Western blot IB:HA Anti-HA抗体Western blot

图4 免疫共沉淀验证EtMIC1与EtMIC2的相互作用

A Cell lysates B Immunoprecipitated complex by Anti-His antibody C Immunoprecipitated complex by Anti-HA antibody 1 pcDNA3.1-His-EtMIC1 and pcDNA3.1-HA-EtMIC2 co-transfection 2 pcDNA3.1-His-EtMIC1 transfection 3 pcDNA3.1-HA-EtMIC2 transfection IB:His Immunoblotting by Anti-His monoclonal antibody IB:HA Immunoblotting by Anti-HA monoclonal antibody

Fig. 4 Identification of the interaction of EtMIC1 and EtMIC2 by Co-immunoprecipitation



A pbFOS-Myc-LC151/pbJUN-HA-KN151 阳性对照组 B 阴性对照组 C pEtMIC1-Myc-LC151/pEtMIC2-HA-KN151 共转染组 DAPI 蓝色荧光通道 Fluor 568 红色荧光通道 Merge 两种荧光与明场叠加效果

图5 双分子荧光互补验证EtMIC1与EtMIC2的相互作用

A pbFOS-Myc-LC151/pbJUN-HA-KN151 positive control group B Negative control group C pEtMIC1-Myc-LC151/pEtMIC2-HA-KN151 co-transfection group DAPI Blue fluorescent channel Fluor 568 Red fluorescent channel Merge Fluorescence and natural superposition

Fig. 5 Identification of the interaction of EtMIC1 and EtMIC2 by BiFc

EtMIC2做双分子荧光试验,激光共聚焦显微镜观察EtMIC1和EtMIC2转染细胞均发出明显的红色荧光,使二者在细胞内的相互作用更加直观。以上试验结果充分证明EtMIC1与EtMIC2在体内外均存在互作,因此推测柔嫩艾美尔球虫微线蛋白可借彼此之间的相互作用帮助虫体主动入侵宿主细胞并得以生存,具体侵入机制仍需进一步研究。

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