Relatório FAPEMIG

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Caracterização bioquímica e molecular de proteínas motoras
tendo como referência a miosina da classe V

1. Apresentação dos dados obtidos da purificação de
miosina-V de cérebro e discussão dos resultados

Neste estudo a miosina-V (BM-V) foi purificada a partir de cérebros de pintainhos
de 1 dia de idade e de nove dias de idade. Grande parte da fração S5 da BM-V foi
solubilizada a partir da fração P4 como mostraram as análise de eletroforese em gel de
poliacrilamida em condição desnaturante com SDS (SDS-PAGE). Nas condições usadas no
tampão da S-500, o resultado obtido no imunoblot ainda sugere que uma pequena parte da
BM-V permanece insolúvel nestas mesmas condições. Este mesmo fenômeno ocorre em
maior grau na solubilização da BM-V durante a homogeneização do cérebro. Acredita-se
que cerca de 40% da BM-V total do cérebro (CHENEY, 1998) permanece associada a
fração particulada de membrana (P1) que não é solubilizada pelas condições de extração.

É importante ressaltar que nossa experiência com 36 preparações de BM-V, ao
longo destes dois últimos anos, apontou que os pontos críticos para se obter uma
preparação com sucesso foram:
- O número de vezes em que se processa a homogeneização, para isto padronizamos em 4
  vezes de 60 segundos cada, mantendo sempre o volume do copo de homogeneização
  completo.
- A potência do homogeneizador também afeta a composição final da fração purificada. Uma homogeneização mais intensa aumenta a composição de proteínas que acompanham a fração purificada. Padronizamos nossa homogeneização para o OmniMACRO Homogeneizer - OMNI.

- O uso de tecido fresco versus o tecido que foi congelado em nitrogênio líquido e conservado a -70°C. Observou-se que a preparação do tecido congelado sempre está sujeita ao aparecimento no SDS-PAGE de bandas adicionais, principalmente a uma dupla banda que migra em ~50 kDa.

Entre as diversas preparações que foram realizadas, o processo foi otimizado de acordo com sugestões de outros trabalhos (TAUHATA, 1999). Nessas preparações notamos que diminuindo o volume dos tampões utilizados ao longo dos passos da purificação o protocolo ficou otimizado em relação ao tempo de execução, economia de reagentes, e quantidade de proteína purificada.

- A qualidade dos reagentes também afetou a qualidade da miosina-V purificada. Isto aconteceu quanto se substituiu o NaCl originalmente usado (SIGMA-S7653) por um NaCl adquirido da REAGEN Química. Neste caso a BM-V purificada quando submetida a ensaio de sedimentação, mesmo a 15.000 g já sedimentava-se na condição controle, indicando desnaturação da proteína causada por algum contaminante presente nesse NaCl. Este problema desapareceu quando voltamos a utilizar um NaCl (SIGMA-S7653), gentilmente cedido pelo Prof. Dr. Luis Cláudio Cameron da UNIRIO.

- A resina de troca iônica “TMAE”, na presença de NaCl, liberou a BM-V mais facilmente que a resina de troca iônica “Q-Sepharose” e foi mais eficaz na eliminação de contaminantes indesejáveis como actina, tubulina, espectrina e debrina. A média das concentrações proteicas obtidas nas preparações foi de 250 μg/ml e as variações observadas foram decorrentes de vários fatores descritos a seguir.

- Com relação à integridade da BM-V aqui purificada podemos observar que ao longo dessas preparações as amostras de BM-V foram utilizadas em vários ensaios de imunoblot como controle de diversos experimentos do laboratório para identificação da BM-V em outros tecidos e organismos. Em adição, não observamos fragmentos de degradação da BM-V mesmo quando ocorreu o armazenamento em geladeira, sob condição tdo tampão de
diálise por mais de duas semanas, tendo sempre o cuidado de adicionar 2mM DTT a cada semana.

- Em nosso laboratório foram utilizadas preparações de BM-V para controle experimental de estudos sobre a identificação de BM-V em glândulas endócrinas e outros tecidos de rato (ESPINDOLA et al. 2000b e Calomys callossus (BARCELOS, 1999; BARCELOS et al., 2000), em sistema nervoso de Apis melifera e Melipona scutellaris (SILVA, 2000, PEIXOTO et al., 2000, BARCELOS, 1999, etc) e em experimentos com macrofágos de murinos J774 (REIS et al., 2001).

A purificação da miosína-V de cérebro (BM-V) de vertebrado tem possibilitado sua caracterização bioquímica. A BM-V purificada em nosso laboratório apresenta um perfil de proteína em eletroforese de gel de poliacrilamida que indica a copurificação das cadeias leves de CaM, cadeias leves do tipo essencial LC23 e LC17 e a cadeia leve homóloga a cadeia leve de dineína e a PIN. Preparações de BM-V em cérebro de pintainhos de um ou nove dias apresentaram o mesmo perfil de cadeias leves. As propriedades da BM-V avaliadas neste estudo sugerem que a proteína preserva suas condições nativas e é propícia aos experimentos aqui realizados. Neste caso, testamos os efeitos de antagonistas de CaM como W7 e melitina sobre algumas das propriedades nativas da BM-V. As implicações dos resultados encontrados para o conhecimento bioquímico, estrutural e funcional desta miosína serão aqui discutidos.

A cabeça trabalhadora de uma miosína inclui um domínio pescoço com funções de regulação e alavanca na transdução mecanooquímica do processo de motilidade, respectivamente (RAYMENT et al., 1993a; XIE et al., 1994). A BM-V está entre as miosinas conhecidas que possui o domínio pescoço mais longo, com seis motivos IQ. Esta é uma das razões em se caracterizar as propriedades estruturais e funcionais do domínio pescoço e as possíveis interações com outros domínios desta miosina.

As atividades enzimáticas e mecânicas da BM-V são parcialmente reguladas pela interação do Ca²⁺ com as cadeias leves de CaM. Esta interação provoca dramáticas mudanças estruturais na molécula da BM-V, com efeitos que variam desde a estimulação da atividade Mg-ATPásica até a diminuição da velocidade de translocação dos filamentos de actina em ensaios de motilidade “in vitro” (CHENEY et al., 1993).
Segundo a revisão de RECK-PETERSON et al. (2000) a purificação de miosina Va resulta na purificação também de cadeias leves de CaM e cadeias leves de 23, 17 e 10 kDa. As cadeias leves de 17 e 23 kDa são as cadeias leves essenciais (ELC) codificadas pelos genes de Gallus galus LC17 e 23, sendo estes também componentes da miosina II não muscular de cérebro (ESPINOLA et al., 2000, NABESHIMA et al., 1987, KAWASHIMA et al., 1987). Se consideramos que a ELC ocupa na BM-V a mesma posição que ocupa na miosina-II podemos supor que a ELC estaria ligada ao primeiro motivo IQ (RAYMENT et al., 1993a; DOMINGUEZ et al., 1998). Em apoio a idéia de que IQ1 tem uma função específica de ligação à cadeia leve essencial, observa-se que IQ1 tem a sequência mais conservada entre todas as miosinas da classe V (com exceção da miosina-V de Drosophila) do que os outros motivos IQ dentro de um mesmo tipo de miosina-V. Além do mais Myo2p de levedura liga-se à CaM e a Mlc1p, um membro da superfamília calmodulina das EF-hand e que provavelmente funciona como uma cadeia leve essencial em levedura (BROCKERHOFF et al., 1994, STEVENS, DAVIS, 1998).

Observamos em nossos estudo que podem ocorrer alterações na estrutura da molécula de BM-V na presença de antagonistas de CaM (melitina e W-7). A BM-V e as cadeias leves de 17, 23 e 10 kDa perdem a solubilidade enquanto as cadeias leves de CaM permanecem solúveis. Utilizamos um peptídeo citoativo do veneno de abelha (Apis mellifera), que exibia excepcionalmente alta afinidade por CaM, para investigar o nível destas alterações.

Os ensaios de solubilidade foram realizados com 100 nM de BM-V e concentrações crescentes de melitina. Os ensaios na presença de ATP foram realizados com 13 μM de cálcio livre e na ausência de ATP com 19 μM de cálcio livre.

Nestes ensaios, na presença e ausência de ATP e com concentrações crescentes de melitina, a BM-V sedimentou-se juntamente com a cadeia leve de 10kDa, enquanto as cadeias leves de CaM permaneceram nos sobrenadantes. Estes resultados foram confirmados através de imunoblot. Assim, evidenciamos que possivelmente as interações de alta afinidade estabelecidas entre as CaM da BM-V e melitina induziram uma mudança de conformação que levou a uma liberação da CaM do domínio pescoço da BM-V com conseqüente alteração da estrutura e agregação observada nesta miosina.
Nas análises densitométricas, observamos que, na presença de ATP, em concentrações de melitina de 0,25 a 1 μM ocorrem modificações na sedimentação da BM-V. Na concentração de 1 μM de melitina, na ausência de ATP, foram observados 59% das amostras no precipitado contra 15% observados nos ensaios na presença deste nucleotídeo. Na concentração de 2,5 μM de melitina evidenciamos a perda mais acentuada da solubilidade da BM-V (92%), na ausência ou presença de ATP. As cadeias leves essenciais de 17 e 23 kDa e a cadeia leve de 10 kDa (LC8/PIN) tiveram sua solubilidade parcialmente alterada ficando, em média, 65% da LC8/PIN, ligada a BM-V, enquanto 90% das cadeias leves de CaM continuaram solúveis.

De acordo com RAYMENT et al. (1993a) no subfragmento S1 de miosina II a ligação do nucleotídeo provoca uma rotação na porção carboxi-terminal da cadeia pesada que é transmitida às cadeias leves possibilitando o movimento. Assim, parece que na miosina II a principal função destas cadeias leves é criar uma molécula maior amplificando as mudanças conformacionais propiciando a ligação do ATP no sítio ativo. Na interação melitina-BM-V este nucleotídeo poderia causar uma mudança de conformação que impediria a melitina de retirar as cadeias leves de CaM da BM-V. Desse modo, na presença de ATP, seria necessário concentrações mais altas de melitina para a insolubilização da proteína.

Os ensaios de cossedimentação na presença de melitina foram realizados na tentativa de verificar o efeito deste antagonista de CaM na interação da BM-V com a F-actina. A cossedimentação foi realizada na presença de 100 nM de BM-V, 1 μM de F-actina e 0,5 e 2,0 μM de melitina. Os ensaios na presença de ATP foram realizados com 13 μM de cálcio livre e na ausência de ATP com 19 μM de cálcio livre.

Como encontramos que a concentração de melitina que causava alteração na solubilidade da BM-V era de 2,5 μM procuramos utilizar valores de concentração que não interferissem na cossedimentação. No entanto, as concentrações de melitina utilizadas (0,5 e 2,0 μM) ou foram insuficientes para verificar seu efeito na interação da BM-V/F-actina ou a melitina não influenciou esta interação. Estes achados nos indicam que a retirada das cadeias leves de CaM da BM-V e as consequentes alterações conformacionais que ocorrem nesta molécula não afetaram o sítio de ligação com a
actina, visto que as cadeias leves de CaM não se cosedimentaram e não impediram a interação da cadeia pesada com a F-actina.

A concentração de melitina (10 μM) utilizada no ensaio de clivagem da BM-V por calpaina não interferiu na proteólise. Os fragmentos obtidos na proteólise da amostra controle foram os mesmos obtidos na presença de melitina e na marcação do imunoblot. Assim, a melitina não interferiu na proteólise da BM-V pela calpaina.

Os ensaios de purificação dos fragmentos cabeça e cauda mostraram, através de imunoblot que o fragmento cauda de 65 kDa foi eluído com 0,15 M de NaCl e o fragmento cabeça com 0,75 M de NaCl. Através de análises de SDS-PAGE também foi verificado que as cadeias leves essenciais foram degradadas pela calpaina e as cadeias leves de CaM permaneceram ligadas ao fragmento cabeça de 65 kDa. Dados de imunoblot revelaram que a cadeia leve de 10 kDa estava ligada ao fragmento cauda.

As preparações de BM-V feitas durante o curso deste trabalho não apresentaram um atividade ATPásica ao nível da relatada em MOOSEKER, CHENEY (1995) que mencionam uma atividade ATPásica de 45-60 ATP/segundo/cabeça de BM-V. O valor máximo de atividade ATPásica ativada por F-actina e estimulada por Ca²⁺/calmodulina foi de 19 ATP/segundos. Em algumas preparações não se observou atividade ATPásica provavelmente devido a desnaturação da miosina-V. Suspeitou-se de vários fatores entre eles, água e pH que estariam afetando a não obtenção de uma BM-V ativa. Com relação a qualidade da F-actina utilizada para estes ensaios, foi padronizado ao longo do desenvolvimento de um controle de qualidade tanto para obtenção do pó-cetônico, utilizando somente acetona de qualidade (Merck). A preparação de G-Actina foi realizada no Laboratório do Dr. Roy Larson (Bioquímica-FMRP). A preparação de G-actina foi testada em vários ensaios de cosedimentação e demonstrada de maneira adequada sua qualidade funcional.

Com relação a atividade ATPase também foram obtidos dados de atividade de uma fração P2 tratada com 0,1% de Triton gelado obtida de acordo com EVANS et al. (1997). A fração P2 enriquecida em vesículas mostrou com sucesso uma atividade ATPásica ativada por F-actina e regulada por Ca²⁺/CaM, inibida por trifluorperazina e não-inibida por 50mM de BDM (EVANGELISTA et al., 1999). Estes dados foram
importantes para confirmar a qualidade dos ensaios de ATPase realizados para BM-V pura tanto em relação à execução do método, qualidade da F-actina e CaM.

Um aspecto importante observado em todas as preparações de BM-V foi a homogeneidade da composição de suas cadeias leves. Um fato a observar é que diferente do que ocorreu nas preparações de BM-V realizadas no laboratório do Dr. Mooseker da Yale University, os pintainhos aqui utilizados são coletadas diretamente na Granja Planalto na mesma manhã de eclosão dos ovos e sacrificados na mesma tarde ou raramente na manhã seguinte, já no caso do laboratório acima referido os pintainhos são comprados por um setor específico da Universidade e geralmente não se tem o controle da idade dos pintainhos.

Em diversas preparações de BM-V realizadas pelo Dr. F. Espindola no Laboratório do Dr. M. Mooseker, notou-se que em alguns casos a cadeia leve de 23 kDa estava ausente. Levantou-se a hipótese de que esta variação poderia ser decorrente da diferença de idade dos pintainhos. Para evidenciar tal fato realizou-se um experimento controle patrocinado pelo Prof. Wilson. Felipe Pereira (ICBM – Anatomia- UFU) que gentilmente cedeu uma gaiola especial, seu laboratório e seus cuidados para a manutenção de 100 pintainhos até a idade de nove dias. O experimento realizado com estes pintainhos em comparação aos pintainhos de 1 dia de idade são mostrados. Observa-se que a cadeia leve de 23 kDa está presente nessa preparação sugerindo que não há variação aparente da composição desta cadeia leve.

A análise densitométrica da BM-V purificada de pintainho de nove dias revela a seguinte composição: para LC 23, CaM, LC 17 e 10 kDa foram respectivamente 1,0; 4,0; 2,0; 3,0 subunidades por cadeia pesada de BM-V. Estes resultados diferem dos valores de 4,0; 0,3; 0,7; 1,0 observados por CHENEY et al. (1993) e ESPINDOLA et al. (2000) para BM-V normal.

Investigamos, também, o efeito do W-7 na molécula da BM-V. Os ensaios de interação foram realizados com 100 nM BM-V e concentrações crescentes de W-7. Os ensaios na presença de ATP foram realizados com 13 μM de cálcio livre.

O efeito do W-7 na solubilidade da BM-V foi observado a partir de concentrações de 60 μM onde observamos 89% da BM-V nas amostras dos sedimentos. Em concentrações de 80 e 100 μM a alteração na solubilidade foi de 93% e 99% e as cadeias
leves de CaM permaneceram no sobrenadante.

A estrutura em solução do complexo Ca\(^{2+}\)/CaM com o antagonista W-7 consiste de uma molécula de W-7 ligando cada um dos dois domínios da CaM (OSAWA et al., 1998). Ensaios de motilidade in vitro, mostraram que a ação de miosina V no deslizamento de actina foi inteiramente inibida por W-7 e em análises de microscopia eletrônica pela técnica de sombreado rotatório foram observadas alterações na estrutura da BM-V submetida a bombardeamento de W-7 na presença de cálcio, possivelmente devido a dissocição das moléculas desta miosina (ESPINDOLA et al., 2000c).

CHENEY et al. (1993) mostraram por visualização em microscopia eletrônica da BM-V purificada um espessamento uniforme da molécula estendendo para trás cerca de 20nm da cabeça globular da miosina. Esta estrutura pode representar a cadeia pesada e as cadeias leves associadas ao domínio pescoço. Ensaios de overlay realizados por ESPREAFCO et al. (1992) mostraram que o domínio pescoço contém os sitos pra ligação de calmodulina. Sequenciamento peptídico das cadeias leves demonstrou que de fato trata-se de calmodulina (ESPINDOLA et al., 2000). A estequiometria dessa associação cadeia-leve calmodulina para a BM-V de cérebro de pintainho de um dia (4-5 calmodulinhas para cada cada cadeia pesada e 1-2 para uma das cadeias leves do tipo essencial) concorda com os dados para a BM-V de cérebro de pintainho de oito dias descartando um possível efeito desenvolvimental na relação da composição destas cadeias leves. Dados obtidos com trifluoperazina, um antagonista hidrofóbico de calmodulina (VEIGAL et al., 1989), mostraram uma inibição da ativação da BM-V pelo Ca\(^{2+}\) na presença de actina (LARSON et al., 1990; ESPINDOLA et al., 1992).

Para NASCIMENTO et al. (1996) a natureza cooperativa da ativação por Ca\(^{2+}\) observada no limite de 1-3µM é remísscente da de outras enzimas reguladas por CaM, confirmando as hipótese de que o Ca\(^{2+}\) atua sobre BM-V via cadeias leves de calmodulina.

Foi mostrado em experimentos de sedimentação que a BM-V incubada com 100 µM de W-7, na presença de cálcio, perdia sua solubilidade. Na tentativa de verificar a possibilidade de recuperação desta solubilidade, o precipitado obtido foi resuspendido em
tampão contendo Imidazol 10 mM, KCl 75 mM, MgCl₂ 2,5 mM, DTT 2 mM e EGTA 1 mM e incubado com calmodulina exógena.

Após a retirada das cadeias leves de CaM pelo W-7 a integridade estrutural da BM-V foi alterada de forma que a ressolfubização não foi possível mesmo com a adição de CaM exógena (a amostra P2 representa a BM-V insolúvel). Já nos ensaios de motilidade "in vitro" a BM-V tratada com o antagonista teve sua solubilidade recuperada após a adição de CaM (ESPINDOLA et al., 2000c) possivelmente devido à diferenças estruturais de conformação entre as moléculas de BM-V em solução e a imobilizada na câmara de motilidade.

OSAWA et al. (1999) investigando as mudanças estruturais na ligação Ca²⁺/CaM na presença de W-7 encontraram que a ligação de duas moléculas de W-7 induziram mudanças estruturais drásticas para o complexo Ca²⁺/CaM que passou de uma estrutura alongada para uma estrutura globular compacta provavelmente pela dobra do ligante flexível. A ligação do complexo Ca²⁺/CaM, nas moléculas de BM-V e na presença de W-7 pode provocar também estas mudanças estruturais comprometendo a atividade fisiológica da proteína.

Entre as questões levantadas em NASCIMENTO et al. (1996) podemos afirmar que algumas delas já foram respondidas e outras ainda não são conhecidas. Isto é a natureza das cadeia leves distintas de calmodulina encontradas por CHENGY et al. (1993) foi revelada como cadeia leves essenciais (ESPINDOLA et al., 2000). As questões se o Ca²⁺ é capaz de dissociar calmodulina? Em que condições? Qual o papel da ligação do Ca²⁺? O que as calmodulinas distantes causariam na regulação do turnover do ATP na cabeça da BM-V?

CAMERON et al. (1998) discutem estas questões. Um dos aspectos é sobre as mudanças conformacionais que ocorrem somente ao nível das cadeias leves para a miosina-II. No caso da BM-V, que tem a CaM como principal componente de cadeia leve, falta o triptofano e a fluorescência da CaM não contribui de maneira significativa para os efeitos que foram observado pelos autores. De acordo com a sequência de aminoácido deduzida do primeiro e quinto motivos IQ de cada cadeia pesada inclui-se um triptofano. Daí a liberação da calmodulina destes sitos IQ poderia potencialmente expor os resíduos do triptofano ao meio aquoso causando decréscimo da fluorescência.
Entretanto, eles não observaram nenhuma mudança do espectro quando Ca\(^{2+}\) foi adicionado, indicando que a exposição desses resíduos de Trp não é a causa primária do quenching da fluorescência. CAMERON et al. (1998) especularam que as mudanças na fluorescência podem ser devido às alterações nas conformações da cadeia pesada que afetam esses e outros resíduos de Trp. Também foi observado que o Ca\(^{2+}\) induz a um decrescimo da \(A_{280}\) do pico que contém a cadeia pesada da BM-V. Dados de outras miosinas revelam que a dissociação das cadeias leves de miosina-II de molusco pode levar a um colapso parcial da região pescoço, de tal modo que se encurta a distância entre a cauda e a cabeça da miosina. Um colapso da região pescoço quando a calmodulina dissocia-se pode correr por analogia com as outras miosinas e pelos dados aqui demonstrados e comentados.

Citando ainda CAMERON et al. (1998) que por comparação com as outras miosinas comentam que os sitios IQ da região pescoço da BM-V são parte de um alfa-hélice longa que é estabilizada pela ligação da calmodulina e de outras cadeias leve, pode se estimar a partir dos dados estruturais que o último sitio IQ em sua extremidade C-terminal está a uma distância de 0.25 nm da região de ligação de actina do domínio cabeça, isto é, cerca de duas vezes mais distantes que na miosina-II de músculo esquelético. Uma mudança conformacional na região pescoço com sub-sequente repercussão na região ligante de actina seria consistente com um mecanismo no qual a ligação do Ca\(^{2+}\) a uma ou mais cadeia leves regularia de algum modo a atividade de um sitio catalítico distante. Desde que a clivagem da BM-V pela calpaina mimetiza a junção cabeça-pescoço, o efeito do Ca\(^{2+}\) em ativar a acto-BM V ATPase, a continuidade da cadeia pesada nesta região deve ser essencial para a regulação.

Um das características básicas da miosina da classe-V é que elas não formam filamentos como a miosina-II. Neste caso a miosina-II possui dois tipos de conformação referidas como formas 6 e 10 S de acordo com seus coeficientes de cossedimentação. A forma 6 S forma filamentos sob condições iônicas fisiológicas o que não é observado na forma 10 S. A falta das cadeias leves de 17 e 20 kDa impede o enovelamento na forma 10 S, resultando na formação de filamentos na presença de ATP. Esta menor habilidade do enovelamento na forma 10 S para as miosina sem as cadeias leves de 20 kDa ou ambas cadeias leves pode ocorrer devido a coesão destas duas cabeças.
O modo de ligação das cadeias leves regulatórias são mostrados no fragmento S1 da estrutura em cristal da miosina-II e possuem características que assemelham-se às estruturas de ligação do complexo Ca²⁺/CaM. A estrutura quaternária do pescoço da miosina muscular envolve não somente interações específicas das cadeias leves com as cadeias pesadas mas também estabilizam o contato do domínio N-terminal da cadeia leve essencial (ELC) com o domínio C-terminal da cadeia leve regulatória (RLC) (RAYMENT et al., 1993a).

Poderia a calmodulina exercer na BM-V o mesmo papel que as cadeias leves regulatórias da miosina II? Sua liberação da BM-V poderia causar a exemplo das RLC na miosina II, perda de conformação nativa e função? Isso seria possível se considerarmos que os motivos IQ da região pescoço sofreriam um possível colapso pela ausência das cadeias leves de CaM, o que propiciaria novas interações com outras regiões da molécula ou com outras moléculas de BM-V. Estas modificações conformacionais iniciadas no domínio pescoço poderiam ser amplificadas para toda a região da cabeça da BM-V causando sua desnaturação.

Existem várias sugestões de diversas funções para a cadeia leve de CaM e outras cadeias leves pertencentes a superfamília das proteínas EF-hand. Tem-se proposto que a ligação das cadeias leves estabiliza o braço em alavanca que é necessário para gerar o tamanho de passo requerido para a velocidade de deslizamento (RAYMENT et al., 1993a, UYEDA et al., 1996, HOWARD, SPUDICH, 1996, METHA et al., 1996). Cepas de leveduras em que estão ausentes os seis motivos IQ da Myo2p, as leveduras crescem quase tão bem quanto as cepas selvagem, sugerindo que a falta do domínio pescoço não é essencial para a função da Myo2p. Por outro lado, leveduras que super-expressam Myo2p crescem muito pouco, mas se super-expressa também a Mlc1p, a equivalente a cadeia leve essencial da BM-V, elas crescem normalmente (STEVENS, DAVIS, 1998).

Segundo RECK-PETERSON et al. (2000) a função primária das cadeias leves na miosina-V de levedura pode ser fornecer um suporte estrutural para o domínio pescoço da Myo2p. Todavia, se o comprimento do domínio pescoço correlaciona com o tamanho do passo, a Myo2p sem pescoço pode não ser capaz de movimento processivo ao longo do microfilamento. Portanto se a Myo2p é um motor processivo como a BM-V, a
viabilidade da Myo2p sem pescoço levanta a possibilidade que sua função seja essencial mas não requeira movimento processivo.

Em eucariontes superiores as cadeias leves teriam papéis regulatórios adicionais aos observados para Myo2p. Visto que a miosina-V de cérebro é regulada por Ca$^{2+}$ in vitro. RECK-PETERSON et al. (2000) sugerem que provavelmente um modo de regulação da miosina-V seja por meio de alteração da rigidez flexional do domínio pescoço tanto pela dissociação da cadeia leve de CaM ou alteração da afinidade de ligação da cadeia leve. Entretanto, pelo fato da motilidade in vitro da miosina-Va ser inibida por Ca$^{2+}$, consideramos que o papel do cálcio na função da miosina-V seja ainda desconhecido (CHENEY et al., 1993, WOLENSKI et al., 1993). As funções essenciais da Myo2p de levedura não devem ser regulada por Ca$^{2+}$ uma vez que as cepas mutantes de calmodulina que falham em se ligar ao Ca$^{2+}$ in vitro são viáveis (GEISER et al., 1991).

Através do uso de inibidores intracelulares da CaM procuramos, com nosso estudo contribuir para a elucidação da complexidade dos detalhes moleculares que envolvem a regulação da BM-V e suas cadeias leves.

As cadeias leves de CaM ligam-se aos motores IQ por interações hidrofóbicas, e como na miosina muscular; estas cadeias leves estariam fazendo interações específicas com as cadeias pesadas. A retirada destas cadeias leves pela melitina e W-7 levariam a molécula de BM-V a perder sua integridade física e estrutural, causando na BM-V uma mudança de conformação com perda de solubilidade e consequente desnaturação.

Como não foram observados efeitos significativos da melitina nas cadeias leves de 17, 23 e 10 kDa, a perda de solubilidade observada parece ter sido decorrente da ausência das cadeias leves de CaM.

Portanto, consideramos que o uso dos antagonistas de CaM constitui uma ferramenta útil para indicar que a BM-V requer as cadeias leves de CaM associadas ao domínio pescoço para manter sua integridade física e funcional.
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2. RELATO DAS ATIVIDADE DE OUTROS TRABALHOS QUE SE DESENVOLVERAM A PARTIR DO PROJETO DE PURIFICAÇÃO DE MIOSSINA-V

2a. COMPOSIÇÃO DE P2-TX E REGULAÇÃO DE SUAS ATPASES POR METAIS E DROGAS: P2-TX, UMA PREPARAÇÃO DE MIOSINA-V DE CÉREBRO ATIVADA POR TRITON X-100, CAPAZ DE MOVER VESÍCULAS NO CITOESQUELETO DE ACTINA

EVANGELISTA, M. L. Composição de P2-TX e regulação de suas ATPases por metais e drogas: P2-TX, uma preparação de miosina-V de cérebro ativada por triton X-100, capaz de mover vesículas no citoesqueleto de actina. 2000. Dissertação (Genética e Bioquímica) - Universidade Federal de Uberlândia, Conselho Nacional de Desenvolvimento Científico e Tecnológico/DF, (Orientador) Foued Salmén Espindola.
Objetivos:
- Utilizar uma preparação de cérebro de pintainho enriquecida em vesículas e membranas que possibilitasse a identificação de componentes do citoesqueleto e de transdução de sinal, através de técnica de imunoblot.
- Investigar as propriedades de atividades ATPásicas desta preparação após tratamento com detergente biológico, em condições que preservassem a integridade das vesículas sinápticas (fração P2-TX).
- Caracterizar a atividade Mg-ATPásica da fração P2-TX em relação ao cálcio e antagonistas de calmodulina.
- Determinar os efeitos de alumínio, fluoreto e do complexo fluoreto de alumínio sobre a atividade Mg-ATPásica de P2-TX.

Conclusões:
- Os imunoblots não indicaram a presença de miosina-II em P2-TX e revelaram miosina-V, miosina-VI, e CaM-kinase-II. Sintaxina e sinaptotagmina foram as proteínas identificadas associadas a vesículas.
- P2-TX tem atividade Mg-ATPásica ativada por F-actina, regulada por cálcio e calmodulina, sendo inibida por trifluoperazina e alumínio. O BDM inibiu parcialmente a atividade de P2-TX.
- P2-TX constitui um modelo para estudo in situ de propriedades das vesículas sinápticas associadas ao citoesqueleto e à transdução de sinal.

3. RELATO DO ENVOLVIMENTO DO LABORATÓRIO DE BIOQUÍMICA E BIOLOGIA MOLECULAR DO INGEB- DA UFU COM PESQUISAS COM ABELHAS, MOTORES MOLECULARES E COLABORAÇÕES.

A identificação estrutural do ponto de vista bioquímico e morfológico do sistema nervoso de *Apis mellifera* aliada à identificação e caracterização de miosinas, em especial miosina-V e suas cadeias leves em neurônios de gânglios nervosos da larva e do cérebro das distintas castas de *Apis* constituem o tema de investigação dos Laboratórios do Prof. Dr. Foued S. Espindola da UFU em colaboração com a Profa. Dra. Enilha E. Espreañico (Departamento de Biologia celular e Molecular FMRS-USP) e Prof. Dr. Antonio Roberto Martins
(Departamento de Farmacologia – FMRP –USP)

Estes estudos foram iniciados em 1999 e já proporcionaram alguns resultados interessantes de identificação e localização de miosina-V no sitema nervoso e reprodutor de Apis mellifera e de uma espécie de abelha brasilífera Melipona scutellaris, muito utilizada nas pesquisas do grupo do Prof. Dr. Warwick E. Kerr em Uberlândia. Nossos esforços de pesquisa podem ser demonstrados pelos dados sobre 3 bolsas de PBIC/CNPq, estar sendo defendido no dia 21/2/2001 a quinta tese de mestrado no período de um ano envolvendo estudos com miosinas, dineínas e pesquisa sobre estas proteínas em Apis mellifera, e dois alunos de doutorado, dois de mestrados e e dois alunos de IC envolvidos nesse projeto.

como orientador de doutorado com o projeto da doutoranda Maria de Fatima Rodrigues da Silva. Aprovação do relatório financeiro do Projeto financiado pela FAPEMIG (CBS 1781/97). Aquisição de vários itens de consumo e permanente através da UFU. Viajem com travel grant do Pew Foundation para Yale University em 11/99 a 01/00. Viajem para o México para apresentação na Reunión dos alumni fellows do Pew na América Latina. Seleção e participação no Curso Intrenacional de sequenciamento Genomico e Bioninformática patrocinada pela Academia de Ciências dos Estados Unidos, financiado pelo Howard Hughes Medical Institute e pelo Centro Brasileiro e Argentino de Biotecnologia. Participação nos dias 11 a 16/12 juntamente com os Profs. Wilson F. Pereira e Tales Alexandre A. Ferreira (doutorandos) no Curso Internacional sobre STEM CELLS patrocinado pela UNESCO - Fundação Calouste Gulbenkian de Portugal realizado na UFRJ (11-15/12/00). Colaborações iniciadas com a Profa.Dra. Carminda da UNESP de Rio Claro para estudar miosina V no sistema reprodutor de abelha, com Prof.Dr. Anotinio Roberto Martins do Departamento de Farmacologia da Faculdade de Medicina da USP de Ribeirão Preto sobre localização de LC8/PIN em retina humana (tese de doutorada defendida e manuscrito em preparação) e para imunolocalização no cérebro de abelha com a Andreia B. Passos Lima, colaboração com a Dra. Enilza Espreafico do Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos da FMRP USP de Ribeirão Preto para a expressão e localização de LC8 em melanócitos em cultura transfectados com LC8 em fusão com GFP (tese de douturamento de Eliana Valória Patussi), localização usando microscopia confocal de miosina V em cérebro de abelha com o mestrandando Pablo Veras Peixoto, para screening de um biblioteca de cDNA de cérebro de Apis Mellifera sem o lobo óptico gentilmente cedida como presente pelo Dr. Gene Robison da University of Illinois, USA através de informação do Dr. Paul Ebert (University Queensland, Austrália) e na qual trabalhavam o mestrandando Pablo M.V. Peixoto para clonagem de miosina-V, a bolsista de IC Camila M teixeira para clonagem da LC8/PIN e a doutorando Maria de Fátima R. Silva para clonagem de miosina-VI. trabalho em colaboração com a Profa.Dra Enilza M Espreafico (USP-Ribeirão) e o Prof. Dr. Jose Roberto Mineo (ICBIM-UFU), que
está sendo desenvolvido pelo doutorando Jair Junior para localização do gene de LC8 a partir de uma biblioteca de cDNA de *Toxoplasma gondii*. Projeto de sequenciamento de proteínas ligante de caiamodulina de *T. gondii* pelo doutorando Prof. Jair P. Cunha Junior em colaboração com o Prof. Dr. Lewis Joel Greene (FMRP-USP). Visita ao LABIBI do Prof. Dr. Blass Lotina Hanssen (UNAM-México) a convite da Profa. Dra. Sandra Furtado (IQ-UFU) para desenvolver trabalho sobre herbicidas naturais de plantas do cerrado. Convenio informal de colaboração com os Apicultores do Apiário Girassol de Uberlandia e Casa do Mel de Governador Valadares para fornecimento de geleia real para o projeto de IC de Inez R. Diaz. Continuidade do fornecimento de pintainhos pela Granja Planalto de Uberlandia para extração e purificação de miosina-V para o trabalho de IC (PBIC-CNPq) Thiago N. França. Estabelecimento de convênio com o laboratório de Neurociências do Prof. Dr. Garcia Delgado da Espanha feita pelo doutorando Prof. Wilson Felipe Pereira (ICBIM-UFU). O investimento na nova linha de pesquisa do LABIBI sobre Bioinformática e suas aplicações ao genoma e proteoma, para introdução como disciplina no curso de graduação e pós-graduação da UFU e como tema de tese da dotoranda M. Fátima R. Silva. Finalmente, todo o desenrolar dos eventos de 2000 que marcaram a nossa entrada no projeto de sequenciamento de ESTs de *Apis mellifera* com a FMRP e a Fundação Hemocentro de Ribeirão Preto e a formação ainda que embrionária da rede genoma UFU com os professores da Faculdade de Computação e Instituto de Genética e Bioquímica.
Myosin V and iNOS expression is enhanced in J774 murine macrophages treated with IFN-γ

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Abstract

Actin-based-motor protein requirements and nitric oxide (NO) production are important features of macrophage activity during phagocytosis or microbicidal processes. Different classes of myosins contribute directly or indirectly to phagocytosis by providing mechanical force for phagosome closure or organelle movement. Recent data have shown the presence of myosins IC, II, V and IXb in phagosomes of bone marrow-derived murine macrophages. In our investigation we demonstrated the presence of different classes of myosins in J774 macrophages. We also analyzed the effect of gamma interferon (IFN-γ), with or without calcium ionophore or cytochalasin B, on myosins as well as on inducible nitric oxide synthase (iNOS) expression and NO production. Myosins IC, II, Va, VI and IXb were identified in J774 macrophages. There was an increase of myosin V expression in IFN-γ-treated cells; iNOS expression was increased by IFN-γ treatment, while calcium ionophore and cytochalasin B had a negative influence on both myosin and iNOS expression, which was decreased. The increase in NO synthesis were reflected by increased iNOS expression. Macrophages activated by IFN-γ released significant amounts of NO when compared to control groups. In contrast, NO production by calcium ionophore- and cytochalasin B-treated cells was similar to that of control cells. These results suggest that IFN-γ is involved in macrophage activation by stimulating protein production to permit both phagocytosis and microbicidal activity.

Key words
- Myosins
- Macrophages
- IFN-γ
- iNOS

Activated macrophages are important effector cells in inflammatory processes and in host defense against microorganisms. Elimination of foreign agents by these cells involves phagocytosis and generation of reactive oxygen species such as nitric oxide (NO). NO is an inflammatory mediator directly related to cell activation (1) that contributes to the death or inhibition of different pathogens as well as tumor cells, NO is derived from a catalytic transformation of L-arginine to citrulline by nitric oxide synthases (NOS). There are three major isoforms of NOS: the constitutively expressed neuronal NOS (nNOS), the endothelially expressed NOS (eNOS) and the inducible NOS (iNOS) (2). Both nNOS and eNOS are regulated by the calcium/calmodulin complex and iNOS is a Ca²⁺-independent enzyme (3,4). However, recent reports have suggested that calcium regulates iNOS expression and NO synthesis in macrophages (1,5,6). Although studies...
have been done in an attempt to address the role of calcium in NO synthesis, this relation remains unclear. Calcium ionophore A23187, a mobile ion carrier that transports divalent cations such as Ca$^{2+}$ and Mg$^{2+}$, reduces NO release and iNOS expression by chondrocytes treated with IL-1 (7). However, it can be used to increase NO synthesis in gamma interferon (IFN-γ)-treated murine peritoneal macrophages, providing evidence that calcium is linked to induction of NO synthesis in macrophages (6).

Several studies have reported the effect of different treatments on the regulation of macrophage-like cells by measurement of NO release or NOS expression (1,3,5,6,8,9). Cytokines such as IFN-γ are able to induce the expression of NOS (3), and thus stimulate the microbicidal activity of macrophages. Moreover, treatment with IFN-γ in combination with lipopolysaccharide (LPS) results in high levels of NO production by macrophages (6,10,11), including the J774 cell line (3,11). There is, however, a regulatory mechanism controlling this activation. Treatment with IL-13 before the stimulus with IFN-γ plus LPS was found to inhibit the release of NO when compared to stimulation with IFN-γ plus LPS without IL-13, suggesting that the activation of NOS by IFN-γ and LPS is controlled by downregulating cytokines (12). Production of NO by macrophages is also inhibited by treatment with cytochalasin B, drugs that inhibit actin filament polymerization (12).

In addition to having a microbicidal activity, macrophages also destroy pathogens after engulfment and enclosure in phagosomes. To perform their phagocytic function, macrophages depend on a motility that is conferred by the dynamic reorganization of the cytoskeleton and the involvement of various motor proteins based on microtubules or actin filaments. Actin-based motor proteins are called myosins and hydrolyze ATP to produce mechanical force (13). More than 13 structurally distinct classes have been identified in many cell types (14). Most of them play important roles in cell motility, vesicle transport, membrane traffic, and phagocytosis. Myosin II has been well characterized as providing support in contractile functions of actin filaments in the cells and may aid vesicle budding from the trans-GoGli networks. Myosins V and VI are implicated in organelle movement and vesicle transport in various organisms. Myosin I participates in endocytic and exocytic membrane traffic (14). In addition to these functions, there is strong evidence that myosins contribute to phagocytosis. In a recent study on erythrocytes phagocytosed by murine bone marrow-derived macrophages, four distinct classes of myosins were identified (myosins IC, II, V and IXb) and located in phagosomes. In addition, myosin IC has been implicated in contractile activity during phagosome formation (13). Therefore, it is possible that myosin production is increased after activation to satisfy the protein requirement during cell motility.

The main objective of the present study was to analyze the effect of IFN-γ in the presence or absence of calcium ionophore or cytochalasin B on myosins and iNOS expression and NO release.

J774 murine macrophages were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 μg/ml penicillin and 100 U/ml streptomycin, in a humidified 37°C/5% CO₂ incubator, for 10 days in 25-cm² flasks. On day 11, cells were added to 96-well plates at a concentration of 5 x 10⁴ cells/200 μl (9) in the same medium.

After 24 h, the culture medium of each well was replaced with 200 μl of fresh medium containing one of the following preparations: a) 5% conditioned medium from L1210 mouse leukemia cells (Río de Janeiro Cell Bank, Río de Janeiro, R.J. Brazil) transfected to produce murine IFN-γ; the concentration
of IFN-γ in the conditioned medium corresponded to 2 U/ml when compared to a standard curve of murine recombinant IFN-γ. b) 10 μM cytochalasin B (Sigma Chemical Co., St. Louis, MO, USA), c) 5 μM calcium ionophore (Sigma), d) IFN-γ plus cytochalasin B or IFN-γ plus calcium ionophore, or e) medium alone, followed by incubation for 24 h at 37°C/5% CO₂. For NO measurement, aliquots of 50 μl from wells of each of the above treatments, as well as a blank and standards with different concentrations of Na₃NO₂, were mixed with an equal volume (50 μl) of Greiss reagent (6). NO production was measured at 570 nm using a microplate reader (Titertek Multiskan Plus; Flow Laboratories International A.S., Lugano, Switzerland). DMEM medium was used as blank and diluent to reduce interference.

J774 monolayers were washed with PBS to eliminate excess FBS and homogenized using a micro-extraction protocol (16). After the addition of 15 μl of ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 μg/ml leupeptin, and 1 mM PMSF, pH 8.0), cells were scraped and transferred to Eppendorf tubes and kept on ice for 1 h to complete cell lysis. Aliquots of 5 μl from each lysate were diluted to 50 μl with water for protein estimation by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

A mass of 15 μg of total protein from each cell extract was separated by electrophoresis on a 5-22% gradient minigel (SDS-PAGE) under reducing conditions and transferred to nitrocellulose membranes. Nitrocellulose sheets were then incubated with monoclonal antibodies against iNOS (Transduction Laboratories, Lexington, KY, USA) or myosin II (Biomedical Technologies, Stoughton, MA, USA), or with affinity-purified rabbit polyclonal antibodies produced against human myosin IC, chicken brain myosin Va, pig myosin VI, and human myosin IXa (a gift from M.S. Moosiker, Yale University). Antibody reactivity was assessed following incubation with horseradish peroxidase-labeled secondary antibodies using a chemiluminescent detection system (ECL: Amersham, Arlington Heights, IL, USA). Densitometric analysis was carried out using the Image Master VSD³ videodocumentation system and software (1996, version 2.0; Pharmacia Biotech Inc., San Francisco, CA, USA). Results were analyzed statistically by the Bartlett test, using the Graphpad Instat TM software (Copyright 1990-1993, v2.02).

Myosins IC, II, Va, VI and IXa were identified in J774 cells with the respective single 130-, 200-, 190-, 145- and 230-kDa bands being recognized by the cited antibodies using previously described methods (13-15). As shown in Figure 1A, this cell line presents a large number of myosins of different classes. These findings are consistent with a recent study in which myosins IC, II, V and IXa were identified in murine macrophages (15). Our results also demonstrate that a single cell type can present numerous myosins. This agrees with Bement et al. (17), who documented the expression of at least a dozen myosins within a given vertebrate cell type. There was no evidence of degradation products, probably due to the method of preparation of a homogenate from fresh cells and to the use of protease inhibitors.

Immunoblots of iNOS and myosins are shown in Figure 1B and the corresponding protein densitometric analysis is shown in Table 1. While the amount of iNOS was increased in the IFN-γ-treated group (66.8%), little expression of this protein was detected (2%) in calcium ionophore- and cytochalasin B-treated cells. iNOS expression was also reduced in groups treated with IFN-γ simultaneously with calcium ionophore (~11%) or cytochalasin B (~75%). In myosin immunoblots we observed that myosin V expression was particularly increased in IFN-γ-treated cells (61%) when compared to control cells (5%). Calcium ionophore or cytochalasin B had a negative influence on
protein expression for most of the myosins. However, myosin V was not found to be inhibited by cytochalasin B. NO production was positively correlated with iNOS expression (Figure IC). Macrophages activated by IFN-γ released significant levels of NO (11.24 ± 1.07 μM) when compared to control groups (2.77 ± 0.48 μM) (P<0.05). In contrast, NO production by calcium ionophore- and cytochalasin B-treated cells (2.23 ± 1.14 μM and 1.68 ± 0.69 μM) was similar to control (P<0.05) (Figure IC). The production of NO was also decreased by calcium ionophore plus IFN-γ treatment (3.83 ± 0.71 μM) when

Figure 1 - Effect of IFN-γ, calcium ionophore (Cal) and cytochalasin B (CytB) on nitric oxide (NO) production and on inducible NO synthase (iNOS) and myosin expression. J774 macrophages (5 x 10⁶ cells/mL) were cultured with IFN-γ alone, or with CytB or with A23187 calcium ionophore. NO production was measured by the method of Gros in macrophage supernatants. iNOS and myosin expression was determined from cell lysates by Western blotting. A. Western blot of J774 lysates to determine the presence of myosin IC, II, Va, VI and IXb. Antibodies specifically recognizing myosin heavy chains (αe) B. Western blot of treated macrophage lysates stained with monoclonal anti-iNOS and anti myosin II antibodies, and with affinity-purified rabbit anti myosin V, Va and VI. Lane 1 represents control (medium only) cell lysates. Lanes 2-6 represent lysates from cells treated with IFN-γ, Cal, CytB, IFN-γ + Cal and IFN-γ + CytB. iNOS 130 kDa recognized by specific monoclonal antibody. IC 130 130 kDa heavy chain of myosin IC, II, Va 200 200 kDa heavy chain of myosin II, Va 190 190 kDa heavy chain of myosin Va, VI 145 145 kDa heavy chain of myosin VI, and IXb 230 230 kDa heavy chain of myosin IXb. C. NO production by treated macrophages. Three samples from each treatment were processed in duplicate (N = 3).

Table 1 - Densitometric analyses from Western blotting of inducible nitric oxide synthase and myosin was IC, II, Va and VI after simulatory or inhibitory treatment.

Control DMEM culture cells, IFN-γ 5% of conditioned medium from L1210 cell-treated macrophages, Cal: 5 mM calcium ionophore-treated cells, Cyt: 10 μM cytochalasin B-treated cells, IFN-γ + Cal: simultaneous treatment with IFN-γ and Cal, IFN-γ + Cyt: simultaneous treatment with IFN-γ and Cyt.

<table>
<thead>
<tr>
<th>Protein percentage from immunoblotting (%)</th>
<th>Control</th>
<th>IFN-γ</th>
<th>Cal</th>
<th>CytB</th>
<th>IFN-γ + Cal</th>
<th>IFN-γ + CytB</th>
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<tr>
<td>INOS</td>
<td>10.93</td>
<td>96.80</td>
<td>2.09</td>
<td>2.04</td>
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<td>IC</td>
<td>22.74</td>
<td>36.01</td>
<td>6.27</td>
<td>8.15</td>
<td>13.27</td>
<td>12.45</td>
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<tr>
<td>II</td>
<td>24.67</td>
<td>22.71</td>
<td>8.55</td>
<td>12.98</td>
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<tr>
<td>Va</td>
<td>5.97</td>
<td>51.12</td>
<td>4.26</td>
<td>16.24</td>
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<td>11.04</td>
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compared to IFN-γ-treated groups (P<0.05). The same was observed in groups treated with cytochalasin B plus IFN-γ (2.58 ± 0.43 μM) (P<0.01). To confirm that the inhibition of iNOS and myosin expression was really due to calcium ionophore or cytochalasin B treatment and not to decreased viability, a dose-dependent assay was devised. J774 macrophages were incubated with different concentrations of calcium ionophore (1.25, 2.5, 5.0 and 10.0 μM) or cytochalasin B (2.5, 5.0, 10.0 and 20.0 μM). Both treatments resulted in 70-80% cell viability, similar to that of control, confirming the specificity of our results.

Elimination of microorganisms by macrophages depends on both phagocytosis and release of toxic agents, such as reactive oxygen and nitrogen intermediates. The present results suggest that IFN-γ is involved in J774 macrophage activation by stimulating either iNOS induction or the production of some myosins to support both processes. This is supported by the observation that myosin expression, mainly myosin V, is increased by IFN-γ as observed for iNOS. Myosin V is one of the myosins implicated in vesicle and organelle transport and its expression in J774 macrophages is sensitive to IFN-γ, suggesting that macrophage activation probably increases myosin V production. Furthermore, the cytochalasin B-treated groups showed an inhibitory effect on protein expression for both iNOS and myosins. This can be explained by the fact that the cytoskeleton is involved in signal transduction events.

In conclusion, our data suggest that IFN-γ may participate in the expansion of the myosin repertoire involved in cell motility, opening new perspectives for a better understanding of myosin involvement in the mechanisms of pathogen clearance by activated macrophages.

Acknowledgments

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The Light Chain Composition of Chicken Brain Myosin-Va: Calmodulin, Myosin-II Essential Light Chains, and 8-kDa Dynein Light Chain/PIN

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Class V myosins are a ubiquitously expressed family of actin-based molecular motors. Biochemical studies on myosin-Va from chick brain indicate that this myosin is a two-headed motor with multiple calmodulin light chains associated with the regulatory or neck domain of each heavy chain, a feature consistent with the regulatory effects of Ca2+ on this myosin. In this study, the identity of three additional low molecular weight proteins of 23-, 17-, and 10 kDa associated with myosin-Va is established. The 23- and 17-kDa subunits are both members of the myosin-II essential light chain gene family, encoded by the chicken L23 and L17 light chain genes, respectively. The 10-kDa subunit is a protein originally identified as a light chain (DLCS) of flagellar and axonemal dynein. The 10-kDa subunit is associated with the tail domain of myosin-Va. Cell Motil. Cytoskeleton 47:269–281, 2000. © 2000 Wiley-Liss, Inc.

INTRODUCTION

The myosin superfamily of actin-based molecular motors consists of at least fourteen structurally distinct classes of unconventional myosins in addition to the well-characterized conventional myosins-II of muscle and nonmuscle cells [Merrell et al., 1998; Wu et al., 2000]. The defining feature of the heavy chain of all myosins is the presence of a structurally conserved N-terminal head or motor domain and, with one known exception [Heintzelman and Schwartzman, 1997], a neck (or regulatory) domain that is followed by a tail domain of varied structure and size, depending on the myosin class. The neck domain is of variable length and consists of one or more IQ motifs, a ~24 amino acid segment, each of which serves as a binding site for a myosin light chain [Houdusse et al., 1996; Mooseker and Cheney 1995]. All known myosin light chains are members of the EF-hand superfamily of proteins. These include the family of genes encoding the essential and regulatory light chains associated with the two IQ motifs that comprise the neck domain of myosin-II. Acanthamoeba myosin-IC contains a novel EF-hand protein structurally

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more similar to the Ca\(^{2+}\) binding protein, calmodulin, than either essential or regulatory light chains [Wang et al., 1997]. Most of the unconventional myosins characterized biochemically thus far utilize one or more copies of calmodulin as light chain [Mooseker and Cheney, 1995]. Recently, however, it was shown that the yeast class V myosin, myo2p, contains both calmodulin light chains and a novel E-F hand light chain termed Mc1p [Stevens and Davis, 1998]. These neck-associated light chains are thought to be involved in motor regulation as well as maintaining the structural integrity of the helical neck domain, which connects the head and tail domain [Houdusse et al., 1996; Lowey and Trybus, 1995; Raymond et al., 1993; Ruppel and Spudich, 1996]. In conjunction with motor regulation, it has been proposed that the neck, with its associated light chains, may serve as a lever arm of defined length for force transduction, thus defining step size as myosin moves along the actin filament during each cycle of ATP hydrolysis [Highsmith, 1999; Howard, 1997; Huxley, 1998; Uyeda et al., 1996].

Myosin-Va is among the best characterized calmodulin-containing myosins [reviewed in Reck-Peterson et al., 2000]. The heavy chain of myosin-Va is encoded by one of three known vertebrate class V heavy chain genes [Mercer et al., 1991; Rodriguez and Cheney, 1998; Zhao et al., 1996]. In mouse, myosin-Va is encoded by the dilute gene [Mercer et al., 1991] and the availability of mutant dilute alleles has facilitated functional analysis. Myosin-Va functions revealed from such analyses implicate this myosin in the transport and/or tethering of several classes of organelles whose primary mode of movement is driven by microtubule motors. This includes a role in transport and/or retention of melanosomes within the dendritic (microtubule-free) processes of melanocytes [Lambert et al., 1998; Nascimento et al., 1997; Provance et al., 1996; Wei et al., 1997; Wu et al., 1997, 1998], transport and/or positioning of smooth endoplasmic reticulum within the dendritic (microtubule-free) spines of Purkinje cells [Dekker-Ohno et al., 1996; Takagishi et al., 1996] and the augmentation of microtubule-based organelle transport within the axon [Bridgman, 1999]. The recent demonstration that chicken myosin-Va, like the microtubule motor kinesin, is processive, is consistent with the role of myosin-Va in organelle movement [Mehta et al., 1999].

Myosin-Va purified from chick brain has been extensively characterized biochemically. It is a two-headed myosin that exhibits robust actin-activated MgATPase and motor activity that are both regulated by Ca\(^{2+}\), presumably via its calmodulin light chains [Cheney et al., 1993; Nascimento et al., 1996]. These calmodulin light chains may also provide structural support for the neck domain, which consists of six IQ motifs and is \(~20\) nm in length [Cheney et al., 1993]. The coordinated action of myosin-Va's two heads, through lever arm rotation of each neck, may produces the large, processive \(~36\) nm steps that have been measured for this myosin [Mehta et al., 1999]. However, analysis of purified chick brain myosin-Va revealed the presence of \(~4–5\) calmodulin/heavy chain, not the \(~6\) predicted from its neck structure [Cheney et al., 1993]. This suggested partial loss of calmodulin might occur during purification and, as a consequence, the observed enzymatic and motor activities may not be entirely reflective of the native molecule. Alternatively, two additional subunits of 17- and 23-kDa co-purify with myosin-Va [Cheney et al., 1993] that might occupy IQ sites on the neck domain. In the course of the present study, we also discovered the presence of a third associated protein of \(~10\) kDa. These additional associated proteins may play critical roles in the structure and function of myosin-Va. Given that current [De La Cruz et al., 1999; Trybus et al., 1999; Wang et al., 2000] and future efforts to provide detailed biophysical and structural characterization of myosin-Va require protein quantities obtainable only by in vitro expression, identification and characterization of the association state of these associated proteins with myosin-Va heavy chain is critical.

In the present study, the identity of the 17-, 23-, and 10-kDa light chains of myosin-Va was determined through peptide sequence and molecular weight analysis. The 17- and 23-kDa proteins are members of the essential light chain family that in class II myosins are bound to the first of the two IQ motifs within the neck domain. Sequence analysis identifies the 10-kDa subunit as a protein that interacts with a diverse array of target proteins. This protein was first characterized as one of the light chains (DLC8) of axonemal and cytoplasmic dynein [King et al., 1996; King and Patel-King, 1995] and subsequently as a potential inhibitor (termed PIN) of the neuronal form of NO synthase [Jaffrey and Snyder, 1996]. DLC8/PIN has also been shown to interact with the transcriptional regulator, \(\kappa\) kappaB alpha [Crepeux et al., 1997] and with the Bcl-2 family member, Bim [Puthalakath et al., 1999]. In the myosin-Va molecule, DLC8/PIN is tightly associated with the tail domain. The identification of these light chains was reported in an earlier preliminary report [Espindola et al., 1996].

**MATERIALS AND METHODS**

**Microsequence Analysis of Chick Brain Myosin-Va Light Chains**

Myosin-Va was purified from 2–3-day-old chick brain tissue as described in Cheney [1998]. To assess whether light chain content of myosin-Va changed as a function of age after hatch, one preparation of myosin-Va
was isolated from 9–10-day-old chicks. The ratio of heavy chain to each of the light chains was determined by densitometry of SDS gels containing a serial dilution of known concentrations of myosin-Va, calmodulin, and bacterially expressed Chlamydomonas DLC8. To concentrate the 10–17-, and 23-kDa light chains, and reduce the amount of calmodulin in the preparation, aliquots of purified myosin-Va, in Buffer A (10 mM imidazole, pH 7.2, 75 mM KCl, 2.5 mM MgCl₂, 2.0 mM dithiothreitol, 0.1 mM ethylene glycol tetra-acetic acid [EGTA]) were heat denatured by placement in a boiling water bath for 3 min. Most of the calmodulin remains in the supernate, while the heavy chain and non-calmodulin light chains can be concentrated as a precipitate by sedimentation. The resulting pellets were run on Bio-Rad (Richmond, CA) pre-cast 4–20% gels and transferred to sequence-grade Immobilon filters (Millipore, Bedford, MA) for subsequent sequence analysis. Analysis of tryptic peptides obtained from the excised light chain bands was performed either by the W.M Keck Foundation Biotechnology Resource Laboratory, Yale University, or the Worcester Foundation for Biomedical Research following methods described in King et al. [1996]. Molecular weights of intact light chains and tryptic peptides were determined by mass spectrometry (matrix-assisted-laser-desorption-ionization time-of-flight) using the facilities cited above at both the Worcester Foundation for Biomedical Research and Yale University.  

Analysis of Head- and Tail Fragments of Myosin-Va Obtained by Calpain Cleavage  

Preparations of myosin-Va (0.1–0.2 μM in buffer A with added 2.0 mM CaCl₂) were digested with calpain as described in Nascimento et al. [1996], which results in the formation of stable heavy chain fragments of 60- and 80-kDa derived from the head and tail domains, respectively; only the 10-kDa light chain and calmodulin light chains remain intact after digestion. To determine with which domain the 10-kDa light chain is associated, several methods, including actin cosedimentation, immunoprecipitation, ion exchange chromatography, and calmodulin affinity chromatography, were employed to obtain separated head and tail fragments. Actin cosedimentation (in the absence of ATP) was performed as described in Nascimento et al. [1996]. The head fragment and associated calmodulin light chains, but not the 80-kDa tail fragment, cosediment with F-actin. Actin was purified from chicken skeletal muscle by the method of Spudich and Watt [1971]. Immunoprecipitation of the tail domain from unseparated calpain digests of myosin-Va was performed by overnight incubation at 4°C with 20 μg/ml of either anti-globular tail myosin-Va or non-immune rabbit IgG (Sigma, St. Louis, MO) in the presence of Buffer A with added 150 mM NaCl and 0.2% Triton X-100 followed by addition of protein A Sepharose beads (Pharmacia, Piscataway, NJ). The bead suspensions were gently mixed using a rocking platform for 30 min at 4°C. The protein A Sepharose beads were collected by sedimentation in a microfuge, supernates saved for immunoblot analysis, and the bead pellets were washed once with 0.5M NaCl in Tris-buffered saline (TBS: 50 mM Tris, pH 7.5, 150 mM NaCl) and then six times in TBS. The washed pellets, together with the initial supernates, were then dissolved in SDS sample buffer and analyzed for 10-kDa light chain and myosin-Va tail fragment content by SDS-PAGE and immunoblot using anti-DLC8 and anti-tail myosin-Va.  

Anion exchange chromatography using Q-Sepharose (Pharmacia, Piscataway, NJ) was performed by applying calpain digests of myosin-Va in Buffer A to ~1 ml column followed by stepwise elution with buffer A containing 0.15 M NaCl, followed by 0.75 M NaCl. Calmodulin affinity chromatography (Pharmacia) was performed by applying myosin-Va calpain digests to the column in Buffer A plus 2 mM CaCl₂, followed by elution with Buffer A plus 5 mM EGTA and then SDS PAGE sample buffer. For all the above fractionations, the presence of the calmodulin and 10-kDa light chains in the various fractions was ascertained either directly by SDS PAGE or by immunoblot using anti-DLC or anti-calmodulin (UBI, Lake Placid, NY). Flagella isolated from Chlamydomonas (gift from the laboratory of Joel Rosenbaum, Yale University) were used as a positive control for the DLC8 blots. All immunoblot analysis was performed by the ECL method following the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN). For some experiments, retention of the 10-kDa light chain on the immunoblot was maximized by incubating the PVDF membranes (0.2 μm sequence grade Bio-Rad) with 0.2% of Glutaraldehyde (8% EM grade solution, Electron Microscopy Science, Fort Washington, PA) in TBS for 40 min, followed by two washes in TBS prior to blocking and antibody incubation using the standard protocol.  

Determination of DLC8/PIN Light Chain Association With Mouse Myosin-Va  

To assess whether the association of DLC8/PIN with myosin-Va is unique to chicken, myosin-Va was purified from mouse brain homogenates by immunoprecipitation. Mouse brain tissue (0.2–0.3 g/m) was homogenized in 5 ml of homogenization buffer [Cheney, 1998] (40 mM Hepes, pH 7.7, 10 mM K-EDTA, 10 mM ATP, 2 mM DTT, and 1 mM AEBSF) using a glass dounce homogenizer. Homogenates were spun at 30,000 g for 20 min, and Triton X-100 and NaCl were added to the resulting supernatant to final concentrations of 0.1% and 150 mM, respectively. Myosin-Va was immunoprecipi-
tated from this supernate using affinity purified polyclonal antibodies (2 μg/ml) raised against the globular tail domain of chicken myosin-Va and protein A Sepharose beads (50 μl of beads/0.5 ml of extract). Control immunoprecipitations were done in the absence of primary antibody. After incubation for 1 h at 4°C, the protein A beads were collected by pelleting through a 1 M sucrose cushion in HSB (0.1% SDS, 0.5% Triton X-100, 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl) and washed sequentially with 1 M NaCl in HSB and low salt wash buffer (10 mM Tris-HCl, pH 7.5). Washed beads and supernates from the immunoprecipitates were dissolved in SDS PAGE sample buffer and assessed for presence of myosin-Va heavy chain and DLC8/PIN by immunoblot analysis as described above.

Assessment of the Association State of the 10-kDa Light Chain With Myosin-Va

To determine the nature of the binding interaction of the 10-kDa light chain with myosin-Va, aliquots of purified myosin-Va were treated with various conditions and bound and free 10-kDa light chains separated by filtration using Microcon 100 filtration chambers (Amicon, Beverly, MA). Conditions included the following: Buffer A with 1.0 mM EGTA, Buffer A with 1 mM CaCl2 and 10 μM mexitillin (Sigma, St. Louis, MO), 10 mM Sodium acetate, pH 5.0 (by addition from a 1.0 M stock), 100 mM Sodium carbonate, pH 11.0 (from a 1.0 M stock), 8.0 M urea (made by addition of solid urea to an aliquot of myosin-Va in Buffer A), and Buffer A containing 0.6 M KI (from a 4.0 M stock). The samples were incubated for 10 min at room temperature, spun in the Microcon separators at 3,000g for 10 min. The flow-through was removed and the retentate was washed two times by addition of Buffer A. The retentate and initial flow-through samples were then analyzed by SDS PAGE and immunoblot analysis using the anti-DLC antibody.

To assess the effect of DLC8/PIN removal on the motor properties of myosin-Va, preparations of DLC8/PIN stripped myosin-Va were compared to control preparations using the gliding filament in vitro motility assay [Kron and Spudich, 1986]. Over 100 velocity measurements were made for each preparation using the Metamorph (Universal Imaging Corp., West Chester, PA) image processing system.

Immunolocalization Analysis

Dissociated dorsal root ganglion (DRG) neurons from 10-day-old chicken embryos were cultured on laminin-coated coverslips as previously described [Suter et al., 1995]. After 16 h the cells were fixed with 4% paraformaldehyde, 60 mM Pipes, 25 mM Hepes, pH 7.0, 5 mM EGTA, 3% sucrose for 1 h at room temperature (RT). After washing with PBS (0.15 M NaCl, 20 mM sodium phosphate, pH 7.0), permeabilization with 0.1% Triton X-100 in PBS for 10 min, additional PBS washing and blocking with 5% bovine serum albumin, 10% normal goat serum, 0.5% gelatin in PBS for 30 min, the cells were incubated with primary antibodies in 1:5 blocking solution/PBS for 1 h at 37°C. After washing, secondary antibody incubation was for 30 min at RT. The following primary antibodies were used: (all at 5-10 μg/ml) rat anti-myosin-Va head [Suter et al., 2000], rabbit anti-myosin-Va tail [Suter et al., 2000], mouse anti-dynein intermediate chain, IC 74 (Mab 74.1, gift of K. Pfister, U. Va.; [Dillman and Pfister, 1994]), and rabbit anti-DLC 8. Secondary Texas Red or fluorescein-labeled antibodies were either from Amersham (Arlington Heights, IL) or Jackson Immuno Research (West Grove, PA). Images were obtained using a Diaphot-300 microscope (Nikon Inc, Melville, NY) with a [100] × 1.4 N.A. objective, an Image Point CCD camera (Photometrics, Tucson, AZ) linked to a Metamorph image processing system.

RESULTS

17- and 23-kDa Light Chains Of Chick Myosin-Va Are Encoded by the L17 and L23 Myosin-II Essential Light Chain Genes

Analysis of purified chick myosin-Va on SDS gels revealed the presence of two proteins of 17- and 23-kDa in addition to the prominent calmodulin light chain band [Cheney et al., 1993] (Fig. 1A). These proteins comigrate with the 17-kDa essential light chains associated with myosin-II purified from gizzard and the 17- and 23-kDa essential light chains associated with myosin-II purified from chick brain (Fig. 1A). Based on densitometry of such gels, it was previously determined that the myosin-Va heavy chain:calmodulin ratio is 1.8 ± 0.7 while the heavy chain: 17- and 23-kDa light chain ratios (using calmodulin as a dye binding standard) were 1.07 and 1.03, respectively, for the several preparations used for quantitation [Cheney et al., 1993].

Microsequence analysis of these light chains was performed with the hope of establishing their relatedness to other known proteins. The light chain region of such gels was electrotransferred to Immobilon paper and the calmodulin, 17- and 23-kDa light chains bands were digested with trypsin. Sequence analysis of a pair of peptides from the presumed calmodulin band (see Table I) exactly matched chicken calmodulin [Simmen et al., 1985] confirming that calmodulin is the major light chain (only indirect assays such as heat stability and Ca2+-dependent gel shift assays had been performed previously). Analysis of two peptides each from both the 17- and 23-kDa light chains revealed 100% identity to corresponding sequences reported for the 17- and 23-kDa
Fig. 1. A: Comparison of the light chains of myosin-Va (lane BM5; migration position of heavy chain is indicated by HC) with those of myosin-II purified from chick brain (lane BM2) and gizzard smooth muscle (lane GM2; gift from the laboratory of D.L. Taylor, Carnegie Mellon University) by SDS-PAGE (Coomassie blue staining). The 23 kDa (23) and 17 kDa (17) light chains of myosin-Va co-migrate with the corresponding essential light chain in brain and gizzard myosin II, while the calmodulin (CaM) light chain of myosin-Va co-migrates with the regulatory light chain of these myosins. The migration position of a previously undetected 10 kDa light chain (10) is also indicated. For sequence analysis of light chains, preparations of myosin-Va were concentrated and partially calmodulin depleted by heat denaturation (lane pBM5) and run on Bio-Rad 4–20% gels. B: Immunoblot analysis of myosin-Va and flagellar axonomes purified from Chlamydomonas reinhardtii (Fl) using either anti- Chlamydomonas DLC (α-DLC) or anti-calmodulin (α-CaM). C: SDS-PAGE analysis of a myosin-Va preparation from 10-day post hatch chicken brain tissue.

essential light chains that are encoded, respectively, by the L17 [Nabeshima et al., 1987] and L23 [Kawashima et al., 1987; Nabeshima et al., 1988] myosin-II essential light chain genes of chicken, both of which are expressed in adult brain (see Table I). Moreover, the molecular weights of five other tryptic peptide peaks for both the 17- and 23-kDa light chains closely match the molecular weights of peptides predicted from the sequence of these two light chains (Table I).

Based on our preliminary report [Espindola et al., 1996] of the identification of essential light chains in chick myosin-Va, Wang and coworkers [Wang et al., 2000] have recently investigated the presence of essential light chains in myosin-Va purified from adult mouse brain. In contrast to our findings, these workers, using immunoblot analysis, failed to detect essential light chains in their preparation. Since L17 and L23 essential light chain expression in the chicken is developmentally regulated [Kawashima et al., 1987; Nabeshima et al., 1988], one possible reason for the difference in light chain content between chicken and mouse myosin-Va may be that our preparations are routinely prepared from 1–2-day post hatch chicks while the mouse myosin-Va preparation was made from adult brain tissue. To address this issue, a preparation of myosin-Va was prepared from 10-day post-hatch chickens; much of brain development is complete by this time [Rogers, 1995]. Both the 17- and 23-kDa light chains were present in this preparation (Fig. 1C).
TABLE I. Microsequence Analysis of Tryptic Peptides (Shown in Bold) From Myosin-Va Calmodulin, 23- and 17-kDa Light Chains and Comparisons of the Observed Masses of Tryptic Peptides From the 23- and 17-kDa Light Chains With Those Predicted for the L23 and L17 Essential Light Chains

<table>
<thead>
<tr>
<th>Light chains</th>
<th>Sequence</th>
<th>Predicted mass (Da)</th>
<th>Observed mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaM</td>
<td>VFDKDNGGYISAELR HVTMNLGEXLDEEVDEMR</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CHKCaM (K00430)1-2</td>
<td>(K) 24EFITFPK30 (K) 24EFKEEIEEFK45 (K) 24FTFKEFKEAFSFLDFR53 (R) 72ALGHNPNAEVLK84 (R) 116OEITFEDFYEGLR128 (R) 126VFKDKEGNYLVGAEELR144 (R) 145HYLVLQGEK153</td>
<td>884 1,455 2,423 1,364 1,528 1,752 996</td>
<td>884 1,457 2,423 1,365 1,528 1,754 997</td>
</tr>
<tr>
<td>LC23</td>
<td>(K) 24EFITFPK30 (K) 24EFKEEIEEFK45 (K) 24FTFKEFKEAFSFLDFR53 (R) 72ALGHNPNAEVLK84 (R) 116OEITFEDFYEGLR128 (R) 126VFKDKEGNYLVGAEELR144 (R) 145HYLVLQGEK153</td>
<td>884 1,455 2,423 1,364 1,528 1,752 996</td>
<td>884 1,457 2,423 1,365 1,528 1,754 997</td>
</tr>
<tr>
<td>CHKM2 L23 (M34990)5</td>
<td>(K) 24EFITFPK30 (K) 24EFKEEIEEFK45 (K) 24FTFKEFKEAFSFLDFR53 (R) 72ALGHNPNAEVLK84 (R) 116OEITFEDFYEGLR128 (R) 126VFKDKEGNYLVGAEELR144 (R) 145HYLVLQGEK153</td>
<td>884 1,455 2,423 1,364 1,528 1,752 996</td>
<td>884 1,457 2,423 1,365 1,528 1,754 997</td>
</tr>
<tr>
<td>LC17</td>
<td>(K) 24EFITFPK30 (K) 24EFKEEIEEFK45 (K) 24FTFKEFKEAFSFLDFR53 (R) 72ALGHNPNAEVLK84 (R) 116OEITFEDFYEGLR128 (R) 126VFKDKEGNYLVGAEELR144 (R) 145HYLVLQGEK153</td>
<td>884 1,455 2,423 1,364 1,528 1,752 996</td>
<td>884 1,457 2,423 1,365 1,528 1,754 997</td>
</tr>
<tr>
<td>CHKM2 L17 (MOCHG24,45)</td>
<td>(K) 24EFITFPK30 (K) 24EFKEEIEEFK45 (K) 24FTFKEFKEAFSFLDFR53 (R) 72ALGHNPNAEVLK84 (R) 116OEITFEDFYEGLR128 (R) 126VFKDKEGNYLVGAEELR144 (R) 145HYLVLQGEK153</td>
<td>1,851 1,400 1,495 1,407 1,700 996</td>
<td>1,851 1,400 1,495 1,407 1,700 996</td>
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<td>CHKM2 L17 (MOCHG64)</td>
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<td>1,851 1,400 1,495 1,407 1,700 996</td>
<td>1,851 1,400 1,495 1,407 1,700 996</td>
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</table>

*For the presumed CaM and both the 23- and 17-kDa light chains (lLC), a pair of tryptic peptides from each light chain was sequenced (shown in bold type), revealing 100% identity with the corresponding sequences of chicken CaM (CHKCaM) and the 23- and 17-kDa essential light chains encoded by the myosin-II essential light chain genes, L23 and L17 (CHKM2 L23 and L17). [Putkey et al., 1983; [Simmen et al., 1985]; [Nabeshima et al., 1988]; [Matuda et al., 1981]; [Grand and Perry, 1983]; [Nabeshima et al., 1987].

TABLE II. Microsequence Analysis Identifies the 10-kDa Light Chain as DLC8/PIN

<table>
<thead>
<tr>
<th>Chicken BMS 10 kDa LC</th>
<th>NADMSEDMQQDAV DIAAYIK</th>
<th>YNPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamy DDC (U19490)4</td>
<td>NADMSEDMQQDAV DIAAYIK</td>
<td>YNPT</td>
</tr>
<tr>
<td>Human (U32944)5</td>
<td>NADMSEDMQQDAV DIAAYIK</td>
<td>YNPT</td>
</tr>
<tr>
<td>Bovine brain DDC3</td>
<td>NADMSEDMQQDAV DIAAYIK</td>
<td>YNPT</td>
</tr>
<tr>
<td>Rat PIN (U66461)4</td>
<td>NADMSEDMQQDAV DIAAYIK</td>
<td>YNPT</td>
</tr>
<tr>
<td>Mouse PIN (4103059)5</td>
<td>NADMSEDMQQDAV DIAAYIK</td>
<td>YNPT</td>
</tr>
<tr>
<td>Rabbit PIN (AF020710, IBQ)3</td>
<td>NADMSEDMQQDAV DIAAYIK</td>
<td>YNPT</td>
</tr>
<tr>
<td>D. melanogaster DYL1 (Q24117)5</td>
<td>NADMSEDMQQDAV DIAAYIK</td>
<td>YNPT</td>
</tr>
</tbody>
</table>

*Sequences of 3 peptides of the myosin-Va (Chicken BMS) 10-kDa c (and corresponding sequences of flagellar and cytoplasmic DLC8 as well as other homologs) present in the database from a variety of species are shown. Numbers in italic indicate position in the primary structure. The accession numbers of each sequence, with ref. number are listed. Chlamy DDC: C. reinhardtii 8 kDa outer arm flagellar dyenin light chain; PIN: protein inhibitor of neuronal NO synthase. [King and Patel-King, 1995]; [Dick et al., 1996]; [King et al., 1996]; [Jaffrey and Snyder, 1996]; [Mount. unpublished data; [Jaffrey et al., 1998], [Torchio et al., 1998].

10-kDa Myosin-Va Light Chain Shares Sequence Identity With the 8-kDa Dynein Light Chain/PIN and Is Present on Both Chicken and Mouse Brain Myosin-Va.

As noted above, SDS-PAGE analysis of purified myosin-Va on 5-20% gels revealed the presence of a third non-calmodulin light chain of ~ 10 kDa that had not been detected on the 5-15% gels used in our earlier studies (Fig. 1A). It is also present in myosin-Va preparations from 10-day post hatch chickens (Fig. 1C). The ratio of heavy chain:10-kDa light chain was estimated by densitometric analysis of Coomassie-blue stained SDS gel lanes containing purified myosin-Va and known amounts of Chlamydomonas flagellar DLC8 as standard. A heavy chain:10-kDa light chain ratio of 1:0.97 ± 0.24 was obtained. Results of sequence analysis of 3 tryptic peptides derived from the 10-kDa subunit indicate that these peptides exhibit 85-100% identity to corresponding sequences of a ubiquitously expressed protein first identified as a dyenin light chain, DLC8 (actual M; 10.3 kDa) [King et al., 1996; King and Patel-King, 1995], and subsequently as an interactor with several other proteins including neuronal NO synthase (termed PIN) [Jaffrey and Snyder, 1996], the transcriptional control protein, 1
kappaB alpha [Crepeix et al., 1997], and the Bcl-2 family member, Bim [Puthalakath et al., 1999]. Alignment of the 10-kDa peptide sequences with corresponding sequences of DLC8/PIN from a number of species is shown in Table II.

Consistent with the identification of the 10-kDa subunit as DLC8/PIN, antibodies raised against the Chlamydomonas DLC8 react strongly with the 10-kDa light chain of myosin-Va (Fig. 1B; see also [Benashski et al., 1997]). Moreover, a sample of myosin-Va subjected to laser desorption mass spectroscopy showed a clear peak with a mass of 10,257 Da, very close to the predicted mass of other vertebrate DLC8/PIN proteins whose sequences have been determined thus far (e.g., 10,365 Da for human DLC8/PIN [Adams et al., 1995]). If the N-terminus is blocked by removal of the N-terminal methionine and acetylation, the expected molecular weight of the human DLC8/PIN would be 10,276 Da.

10-kDa Light Chain Is Associated With the Tail Domain of Myosin-Va

To determine with which myosin-Va heavy chain domain the DLC8/PIN subunit is associated, head and tail fragments of myosin-Va generated by calpain cleavage were analyzed for the presence of this light chain. Limited calpain proteolysis of myosin-Va results in the formation of a stable 80-kDa tail fragment and a 65-kDa head peptide [Nascimento et al., 1996] (see lane dbM5 in Fig. 2). As shown previously [Nascimento et al., 1996], sedimentation of mixtures of F-actin and calpain digests of myosin-Va results in co-pelleting of the 65-kDa head fragment with actin (see lanes 1 and 2, Fig. 2). Note that the calmodulin light chains also cosediment. Since the 65-kDa fragment lacks the neck domain [Nascimento et al., 1996], it is likely that the neck and bound calmodulin light chains remain associated with the head fragment through noncovalent interactions. Unfortunately, both the 23- and 17-kDa essential light chains are cleaved by calpain (compare lanes BM5 and dbM5 in Fig. 2), so their association with the neck domain could not be verified by this method. In contrast to calmodulin, the 80-kDa tail fragment remained in the supernate after cosedimentation (lane 1, Fig. 2). The DLC8/PIN also remains in the supernate fraction (lane 1', Fig. 2).

Several experimental strategies were employed to determine if the 10-kDa subunit is bound to the 80-kDa tail fragment. Immunoprecipitation of the 80-kDa tail fragment using globular tail domain-directed antibodies from calpain digests (Fig. 3) resulted in co-immunoprecipitation of the DLC8/PIN. Second, separation of head and tail fragments was performed using anion exchange chromatography (Fig. 4). The 80-kDa tail domain eluted from such columns at lower salt concentrations than the 65-kDa head fragment and calmodulin (Fig. 4A-C). Immunoblot analysis reveals that the DLC8/PIN co-eluted from this column with the tail fragment (Fig. 4D). Similar separation of head and tail fragments was obtained by both calmodulin affinity and cation exchange chromatography. In both cases, the DLC8/PIN subunit co-eluted with the 80-kDa tail fragment (results not shown).

Dissociation of the 10-kDa Subunit From Myosin-Va Requires Harsh Solution Conditions

Various solution conditions were tested to assess the association state of DLC8/PIN with myosin-Va.
heavy chain (Fig. 5). Bound and free DLC8/PIN were separated by microfiltration using a filtration apparatus with a pore exclusion limit of 100 kDa. Treatment with the calmodulin inhibitor, mellitin, which results in dissociation of the calmodulin light chains, does not release DLC8/PIN. Complete release of DLC8/PIN was achieved in the presence of 0.1 M sodium acetate buffer at pH 5.0 or 0.1M sodium carbonate buffer at pH 11.0. Partial dissociation was effected by treatments with 0.6 M KI and 8.0 M urea. Only acetate treatment yielded selective dissociation of the DLC8/PIN relative to retained calmodulin light chains. Interestingly, such preparations of DLC8/PIN stripped myosin-Va retain motor activity as assessed by the sliding filament assay. Comparable velocities for both acetate-treated (306 nm/s ± SEM 16.2 nm/s) and control preparations (306 nm/s ± SEM 8.7 nm/s) of myosin-Va were observed indicating that the pH 5 acetate treatment does not denature myosin-Va and that the DLC8/PIN is not required for motor activity.

**Mouse Myosin-Va Contains DLC8/PIN**

To assess whether DLC8/PIN is associated with myosin-Va from other vertebrate species, myosin-Va was isolated by immunoprecipitation from mouse brain homogenates. Immunoblot analysis revealed that DLC8/PIN does co-immunoprecipitate with mouse brain myosin-Va (Fig. 3D,E). Assuming a heavy chain:DLC8/PIN ratio of 1:1, densitometric analysis of quantitative immunoblots of serially diluted supernate and pellet fractions from such immunoprecipitation experiments revealed that only ~3% of the total DLC8/PIN present in the brain extract used for immunoprecipitation is associated with myosin-Va.
Fig. 4. Separation of myosin-Va head and tail domains by Q-Sepharose chromatography. A: SDS-PAGE (Coomassie blue stained) of intact myosin-Va (lane 1), the calpain digest (lane 2), and fractions eluted from the column by 0.15 M NaCl (lane 3) and 0.75 M NaCl (lane 4). Migration positions of the myosin-Va heavy chain (HC), 80-kDa tail fragment (80), 65-kDa head fragment (65), calmodulin (CaM), and the 23-, 17-, and 10-kDa light chains are indicated (23, 17, and 10). B-D: Immunoblot analysis of gels identical to that in A, using antibodies to the globular tail (B; α-tail), head (C; α-head) domain, and anti-Chlamydomonas DLC (C; α-dlc). Note that DLC8/PIN coelutes from the column with the 80-kDa calpain-derived tail fragment.

Myosin-Va Partially Co-Distributes With DLC8/ PIN But Not Dynein Within the Growth Cones of Cultured Neurons

The localization of myosin-Va, DLC8/PIN, and cytoplasmic dynein was examined in cultured DRG neurons from chick embryos. Dynein localization was determined using an antibody raised against the 74-kDa intermediate chain (DIC 74) of cytoplasmic dynein [Dillman and Pfüster, 1994]. All three proteins were distributed throughout these neurons, exhibiting generally punctate staining in cell bodies, neurite shafts, and growth cones (results not shown). With respect to myosin-Va, these results were comparable to those reported previously for both chick and rodent neurons [Espreca et al., 1992; Bridgman 1999; Suter et al., 2000; Evans et al., 1997]. Because of staining intensity and dense packing of puncta containing myosin-Va elsewhere in these neurons, the peripheral domain of the growth cone was the best region to compare the distribution of myosin-Va with the localization of DLC8/PIN and dynein (Fig. 6). Co-staining with anti-Chlamydomonas DLC8 revealed some overlap in distribution with myosin-Va in the growth cone (Fig. 6A–C). However, the coincidence of staining was not complete, as one would expect since the DLC8/PIN interacts with multiple proteins including dynein. DIC 74 staining was qualitatively similar to that of myosin-Va in that within the growth cone, a punctate distribution was observed; however, much less overlap with myosin-Va staining, compared to that with the DLC8 antibody, was seen (Fig. 6D–F). Thus, one potential function for DLC8/PIN, to target both motors to the same cargo vesicles via a common linker complex, is not supported by these observations.

DISCUSSION

The presence of a single essential light chain/heavy chain is a ubiquitous feature of all known class II myosins that have been biochemically characterized. The precise function of the essential light chain is unknown, although its presence is required for myosin-II activities [Lowey and Trybus, 1995; Ruppel and Spudich, 1996]. It seems likely that its association with the most proximal IQ site in the neck domain serves to maintain the structural integrity of this portion of the heavy chain [Lowey and Trybus, 1995; Ruppel and Spudich, 1996; Trybus, 1994]. Presumably, the essential light chains of myosin-Va serve a similar function within the neck domain of this unconventional myosin. However, because calpain
digestion results in cleavage of both the 17- and 23-kDa light chains, we can only assume, albeit reasonably, that these light chains are associated with the neck domain. Key unresolved questions concerning the role of the essential light chains include the following: Do the essential light chains of chicken myosin-Va bind to the most proximal IQ site? Recent analysis of in vitro-expressed chicken myosin-Va constructs consisting of the head and first IQ motif bind either calmodulin or essential light chain, and no significant enzymatic differences were reported [De La Cruz et al., 1999]. Do populations of myosin-Va molecules consist of heterodimers or homodimers with respect to the 17- and 23-kDa light chains and is there a single essential light chain/heavy chain? Does calmodulin/essential light chain composition vary in different cell types of the brain or during embryogenesis? In this regard, we have purified myosin-Va from dissected cerebellum and cortex, and observed no difference in light chain content (Espindola and Mooseker, unpublished observations). As noted above, it has recently been demonstrated that mouse myosin-Va lacks essential light chains. We demonstrate that this is unlikely to be due to the difference in developmental age of the brain tissue used for isolation (adult mice vs. 1-2-day-old chickens) since the essential light chains are present in myosin-Va purified from 9-10 day post hatch chickens. This raises the interesting possibility that their differing light chain content may contribute to differences in mechanochemical activity. For example, chicken myosin-Va is a processive motor, while Wang and coworkers report that mouse myosin-Va, while exhibiting very high affinity for actin like chicken myosin-Va [Nascimento et al., 1996], may not be processive [Wang et al., 2000]. However, such differences also
could be contributed by the respective heavy chains, since although highly homologous (motor domains are ~94% identical), sequence identities in key regions including the region of actin contact (surface loop 2 in myosin-II) and the first IQ motif, the putative essential light chain binding site, show less identity (83 and 78% identical, respectively).

In considering functions for DLC8/PIN within the myosin-Va molecule, the demonstration of its association with motor proteins may be misleading since as noted previously, this highly conserved, ubiquitously expressed protein may interact with a functionally diverse array of binding partners [Crepieux et al., 1997; Jaffrey and Snyder 1996; Putalakath et al., 1999]. However, significant insights into how this small protein may interact with such a wide range of target proteins has been provided by recent analyses of DLC8/PIN structure [Fan et al., 1998; Liang et al., 1999] and oligomerization state. Crosslinking studies indicate that DLC8/PIN is a dimer in both dynein and myosin-Va [Boneshski et al., 1997]. The structure of the DLC8/PIN dimer, with bound peptides from the PIN binding domain of neuronal NO synthase has recently been solved by X-ray diffraction [Liang et al., 1999]. The monomer consists of a highly polarized molecule, with one face consisting of a pair of alpha helices (solvent facing) and the other a largely hydrophobic beta sheet consisting of 5 beta strands. This sheet constitutes the dimerization domain, and, within the dimer, one of the beta strands in each monomer is contributed by the opposing monomer, presumably stabilizing the dimer. The dimer contains two NO synthase peptide binding clefts at either side of this dimerization domain. From the sequence of the NO synthase peptide that binds to this cleft, a consensus sequence for DLC8/PIN binding has been suggested (Asp-Thr-X-Gln-Val-Asp-X) and similar sequences have been identified in the dynein intermediate chain [Liang et al., 1999]. These authors also incorrectly identify a similar sequence in the tail domain of myosin-Va (the sequence identified is actually from a myosin-II). No similar sequence is present in the myosin-Va heavy chain. Nevertheless, like the target peptide binding domain of calmodulin, these hydrophobic binding clefts might interact with a wide range of peptide segments that lack primary structure similarity. Based on these structural studies, one possible function for DLC8/PIN is to stabilize heavy chain-heavy chain interactions/structure within the globular tail of myosin-Va. Consistent with this idea is the observation that each myosin-Va molecule may contain a single DLC8/PIN dimer, as estimated by gel densitometry. Moreover, electron microscopy of myosin-Va molecules indicates that the distal globular tail domain is generally not bi-lobed, suggesting significant stabilization of chain-chain interactions in this region of the molecule [Cheney et al., 1993]. If the primary function for DLC8/PIN is to link the myosin-Va molecule to other target proteins (e.g., cargo docking components), then one might expect there to be one dimer/heavy chain, not molecule. However, densitometric estimates are just that, and some DLC8/PIN may be lost from myosin-Va during purification.

One intriguing potential "cargo binding" function for DLC8/PIN in myosin-Va is that it may tether the motor to particular mRNA transcripts. This possibility is raised by the recent finding that DLC8/PIN binds to the 3' UTR of parathyroid hormone mRNA, a transcript that is localized to the microtubule cytoskeleton [Epstein et al., 2000]. Thus, both cytoplasmic dynein and myosin-Va could play a role, via their respective associated DLC8/PIN light chains, in the localization and/or transport of specific mRNAs.

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Localization of Unconventional Myosins V and VI in Neuronal Growth Cones

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ABSTRACT: Class V and VI myosins, two of the six known classes of actin-based motor genes expressed in vertebrate brain (Class I, II, V, VI, IX, and XV), have been suggested to be organelle motors. In this report, the neuronal expression and subcellular localization of chicken brain myosin V and myosin VI is examined. Both myosins are expressed in brain during embryogenesis. In cultured dorsal root ganglion (DRG) neurons, immunofluorescence of myosin V and myosin VI revealed a similar distribution for these two myosins. Both are present within cell bodies, neurites and growth cones. Both of these myosins exhibit punctate labeling patterns that are found in the same subcellular region as microtubules in growth cone central domains. In peripheral growth cone domains, where individual puncta are more readily resolved, we observe a similar number of myosin V and myosin VI puncta. However, less than 20% of myosin V and myosin VI puncta colocalize with each other in the peripheral domains. After live cell extraction, punctate staining of myosin V and myosin VI is reduced in peripheral domains. However, we do not detect such changes in the central domains, suggesting that these myosins are associated with cytoskeletal/organelle structures. In peripheral growth cone domains myosin VI exhibits a higher extractability than myosin V. This difference between myosin V and VI was also found in a biochemical growth cone particle preparation from brain, suggesting that a significant portion of these two motors has a distinct subcellular distribution.

Keywords: myosin V; myosin VI; neuronal growth cone; dorsal root ganglia neurons; cytoskeleton; subcellular localization

The myosin superfamily consists of at least 15 structurally distinct classes of actin-based molecular motors (Mermall et al., 1998; Probst et al., 1998; Wang et al., 1998). In addition to the well-defined roles of conventional or class II myosins in mediating actin-based contractile phenomena in muscle and non-muscle cells, a wide range of functions have been proposed for various "unconventional" members of

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this gene family. Based largely on genetic and molecular genetic evidence, these functions include organelle movement, endo- and exocytosis, transport of mRNA, signal transduction, and mechanoregulation of ion channels (Mermall et al., 1998). In vertebrates, at least eight (I, II, V, VI, VII, IX, X, XV) of these classes (most of which have multiple members) are expressed. In the vertebrate nervous system, representatives of most of these myosin classes have been identified (reviewed in Hasson and Mooseker, 1997). In this study we focus on the neuronal expression of two myosins of these classes, V and VI, whose members have been implicated in actin-based organelle transport in a variety of systems (Mermall et al., 1998).

There are three known class V myosin heavy chain genes expressed in vertebrates (Merce et al., 1991; Espinofico et al., 1992; Bement et al., 1994; Hasson et al., 1996; Zhao et al., 1996; Rodriguez and Cheney, abstract, Mol Biol Cell 1998, 9, 20a) only one of which, brain myosin V, has been extensively characterized both genetically and biochemically (reviewed in Mooseker and Cheney, 1995; Titus, 1997). Unlike other known myosins, the myosin V heavy chain consists of an amino-terminal motor or head domain, a neck or regulatory domain consisting of six IQ motifs, each a site for light chain binding, and a C-terminal tail consisting of a proximal "stalk" domain comprised of coiled-coil forming alpha helical segments, followed by distal globular domain. The subunit composition of myosin V is the most complex known myosins: it consists of two heavy chains, each of which has several neck-associated light chains, ~5 of which are calmodulin, one of which is an essential light chain also present in brain myosin II and a tail-associated light chain that is a subunit of dynein (Espinofico et al., abstract, Mol Biol Cell 1996, 7, 372a; Benashski et al., 1997). Very recently, myosin V has also been shown to bind and activate Cα2 kinase I, probably delivering calmodulin to the kinase (Costa et al., 1999). In vitro assays revealed that purified myosin V is a Ca2* -regulated, barbed end-directed motor (Cheney et al., 1993) that binds F-actin with high affinity in the presence of ATP (Nascimento et al., 1996).

There is now broad biochemical, cytological, and genetic evidence that myosin V may be an important organelle motor (for reviews, see Titus, 1997; Mermall et al., 1998). For example, it has been demonstrated that myosin V associates with small organelles in growth cones of rat superior cervical ganglion neurons (Evans et al., 1997) and that myosin V can act as an actin-based vesicle motor (Evans et al., 1998; Tabb et al., 1998). Analysis of myosin V in dilute

mice, which carry mutations in the myosin V gene (Merce et al., 1991), provides further evidence that this motor is involved in organelle movement in Purkinje cells (Takagishi et al., 1996) and melanocytes (Provan et al., 1996; Nascimento et al., 1997; Wu et al., 1997; Rogers and Geifland, 1998; Wu et al., 1998).

In contrast to myosin V, myosin VI is less well characterized, especially with respect to its biochemical properties, expression pattern, and potential roles in actin-based motility. The myosin VI heavy chain is a ~140-kDa protein with a typical amino-terminal head domain containing actin- and ATP-binding activity, a short neck with one IQ motif, and a relatively short tail domain with a coiled-coil region (Keller et al., 1992; Hasson and Mooseker, 1994; Avraham et al., 1995, 1997; Buss et al., 1998). Interestingly, it was found that a mutation of myosin VI gene is responsible for the Snael's waltzer phenotype in mouse, a form of severe hearing loss and vestibular dysfunction (Avraham et al., 1995). Immunofluorescence studies of myosin VI in the inner ear revealed that this unconventional myosin is concentrated in the cuticular plate of the hair cells, suggesting that myosin VI may have a role in attachment of stereocilia rootlets to the cuticular plate (Hasson et al., 1997).

Besides this more static function for myosin VI, there is also evidence for involvement of this motor in organelle transport, since myosin VI was localized in the pericuticular necklace, a vesicle-rich compartment of inner ear hair cells (Hasson et al., 1997). Furthermore, functional and localization studies in the Drosophila embryo suggest a role for myosin VI as a transport motor for cytoplasmic particles (Mermall et al., 1994; Mermall and Miller, 1995; Bohrman, 1997; Lantz and Miller, 1998). Myosin VI has also been shown to interact with a glucose transporter binding protein GLUT1CBP, which was implicated as a linker between the motor protein and transporter carrying vesicles (Bunn et al., 1999). Finally, a recent report has provided evidence that myosin VI may have a function in membrane ruffling and membrane traffic pathways in fibroblasts (Buss et al., 1998).

To gain more insights into the neuronal functions of myosin V and myosin VI, we investigated their distribution in cultured chick dorsal root ganglion (DRG) neurons, focusing on their localization in the growth cone. Our immunolocalization experiments revealed a punctate staining pattern in the growth cone for both myosins, partially colocalizing with each other. Despite these similarities, localization and fractionation data suggest that a significant fraction of these two motors have a distinct subcellular distribution in growth cones.
MATERIALS AND METHODS

Antibodies

The following affinity-purified antibodies to myosin V and myosin VI were used in this study: rabbit anti-myosin V-tail; rabbit anti-myosin V-head (both described in Espeset et al., 1992); rat anti-myosin V-head; rabbit anti-
myosin VI-tail (Hasson and Mooseker, 1994). Monoclonal antibodies to β-tubulin and GAP-43 (clone GAP-7B10) were purchased from Sigma Chemical Co. (St. Louis, MO).

Western Blot Analysis of Myosin V and Myosin VI Expression

Chick embryos at different stages throughout embryonic development were used for analysis of myosin V and myosin VI expression in DRG and total brain: stage 29 [embryonic day 6 (E6)], 33 (E8), 36 (E10), 38 (E12), 42–43 (E16), and 45 (E18). Total brain and DRGs were washed with phosphate-buffered saline (PBS) containing 1 mM Pefabloc (Boehringer Mannheim, Indianapolis, IN) and homogenized in 5% cold trichloroacetic acid (TCA). After a spin at 14,000 × g for 10 min, the pellets were first resuspended with 100 mM Tris-HCL pH 8.8, and then were boiled in sample buffer. Equal protein amounts were separated on a 4–20% polyacrylamide gel. Proteins were transferred onto nitrocellulose and probed with rabbit anti-myosin V-tail, rabbit anti-myosin V-head, and rabbit anti-
myosin VI-tail antibodies. Detection was carried out by the ECL method according to the manufacturer’s instructions (Boehringer Mannheim).

Cell Culture

Dissociated DRG neurons from 10-day-old chicken embryos were cultured on laminin-coated coverslips in Dulbecco’s modified Eagle’s medium-F12, 5% horse serum, 2% chicken serum (all from Life Technologies, Frederick, MD), 14 mM NaHCO3, 50 mM Hepes, pH 7.4, penicillin-
streptomycin-fungizone 1:100 (JRH BioScience, Lenexa, KS), and 20 ng/mL NGF. Coverslips were acid- and ethanol-washed and precoated with 20 μg/mL poly-l-lysine in H2O for 30 min at room temperature (RT). After three washes with H2O, poly-l-lysine–coated coverslips were air-dried and stored until they were used for culturing cells. Prior to plating dissociated DRG neurons, the coverslips were coated with 80 μL of 20 μg/mL laminin (Life Tech-
nologies) in PBS for 2 h in a humidified 37°C incubator followed by two PBS washing steps. DRGs were collected into ice-cold Puck’s saline and dissociated by trypsin treatment and trituration using a Pasteur pipette. About 15,000 DRG cells were plated per coverslip in the medium described above and typically were maintained for 16–20 h in a humidified 37°C incubator (5% CO2) before use.

Immunocytochemical Stainings

After a 16 h incubation at 37°C, the cells were fixed with warm fixation buffer: 4% paraformaldehyde in 60 mM PIPES, 25 mM HEPS, pH 7, 5 mM EGTA. 5% sucrose (PHEM buffer) for 1 h at RT. After three washes with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at RT. Following additional PBS washing and blocking with 5% bovine serum albumin (Sigma), 1% normal goat serum, 0.5% gelatin (Sigma) in PBS for 30 min, the cells were incubated with primary antibodies in 1:5 blocking solution/PBS for 1 h at 37°C. All myosin V and myosin VI antibodies were used at 5–10 μg/mL. The monoclonal mouse anti-β-tubulin antibody (Sigma) was incubated at 1:200. After washing with PBS, secondary antibody incubation was at 1:200 for 30 min at RT. Secondary Texas Red- or FITC-labeled donkey antibodies were purchased either from Amersham (Princeton, NJ) or Jackson ImmunoResearch (West Grove, PA). For visualization of F-actin structures, rhodamine-phalloidin (Molecular Probes, Eugene, OR) was used at 1:100. Coverslips were mounted on glass slides using Citifluor (Citifluor Products, Canterbury, UK) as an antifading agent. Cells were examined on an inverted microscope (Nikon, Melville, NY) using a 100×/1.3 NA objective. Images were acquired with a cooled CCD camera (Image Point, Photometrics, Tucson, AZ) controlled by Metamorph software (Universal Imaging Corporation, West Chester, PA). Image processing was carried out in Adobe Photoshop 4.0 (Adobe Systems Incorporated, San Jose, CA). For quantification of puncta, we analyzed color overlays of myosin V and VI double stainings, using the manual count object tool of Metamorph. Single- and double-labeled puncta were counted only in the peripheral growth cone domain where individual puncta could be more readily resolved. Average values ± S.E.M. are given. Student’s t-tests were performed to analyze differences between data sets.

Live Cell Extraction

For live cell extraction experiments, DRG cell cultures were treated with 0.02% of saponin in PHEM buffer for 30 s immediately followed by fixation with 4% paraformalde-
hyde in PHEM buffer containing 1% Triton X-100 for 10 min. The fixation process was completed using the fixation solution described above in the absence of any detergent for 50 min. To investigate structural changes during the live cell extraction process, the cells were observed using high-resolution, video-enhanced differential interference contrast (DIC) microscopy. Therefore, the cells were mounted into a custom-made chamber, kept at 34°C, and inspected on Zeiss Axiosvert-10 microscope (Zeiss, Thornwood, NY) using a 63×1.4 NA oil immersion objective. Image acquisition and processing were carried out as recently described (Suter et al., 1998).
Subcellular Fractionation with Differential Centrifugation

Total brains from 2 chick embryos (E10) were homogenized with the same buffer used for the live cell extraction experiment (PH2M, 0.02% saponin) containing 0.2 mM dithiothreitol (DTT), 1 mM Pefabloc, and 5 μg/mL leupeptin (Sigma). Cell lysis was verified by light microscopy. The low-speed spin was carried out at 1000 × g for 20 min, the high-speed spin at 30,000 × g for 20 min, and the ultracentrifugation at 120,000 × g for 2.5 h. Protein determination was done according to the BCA method (Pierce Chemical Co., Rockford, IL). Stoechiometric amounts of each fraction were separated on a 5–20% SDS-PAGE and blotted to nitrocellulose membrane. Blots were probed with rabbit anti-myosin V-tail and anti-myosin VI-tail antibodies at 0.5 μg/mL each. Detection was carried out with the ECL method according to the manufacturer’s instructions (Boehringer Mannheim). Metamorph software (Universal Imaging Corporation) was used for blot quantification.

RESULTS

Myosin V and Myosin VI Are Expressed in the Chick Nervous System during Embryonic Development

To investigate the expression pattern of myosin V and myosin VI in the developing chick central and peripheral nervous system, we carried out Western blot analysis of whole brain and DRG extracts at different stages of embryonic development (Fig. 1). Using affinity-purified antibodies to myosin V and myosin VI, both unconventional myosins were detected in chick brain between embryonic days 6 and 14, respectively (Fig. 1(B)). The presence of the myosin VI heavy chain was revealed with a rabbit antibody to the tail of myosin VI, whereas the myosin V heavy chain was detected by two distinct rabbit antibodies to the tail and head of myosin V. The level of myosin VI expression in the brain remains constant between E6 and E14. In contrast, brain myosin V expression increases during this time period. In DRGs, we found an increase in both myosin V and VI expression at stage E10, a time period in development when the DRGs increase in size [Fig. 1(B)]. To summarize, both myosins are expressed in the chick central and peripheral nervous system in a similar pattern during embryonic development.

Myosin V and Myosin VI Are Expressed in Cultured Chick DRG Neurons

Dissociated chick DRG neurons of E10 were cultivated on laminin substrate for immunolocalization of myosin V and myosin VI. Both myosin V and myosin VI were detected in neuronal cell bodies, neurites, and growth cones [Fig. 2(A,B)]. DRG neurons exhibit a variety of growth cone morphologies on laminin substrate: from fanlike growth cones [Fig. 2(C)] to the ones with large filopodia but small lamellipodia [Fig. 2(A)]. One can distinguish between two major cytoplasmic domains in neuronal growth cones: the central, microtubule-rich domain and the peripheral, F-actin-rich domain [Forscher and Smith, 1988; see Fig. 2(D)]. In general, both myosins exhibit a punctate staining pattern in growth cones, with a higher concentration of puncta in the central domain compared to the peripheral domain [Fig. 2(C,D)]. Double stainings revealed that 17 ± 2% of the myosin V and 18 ± 3% of the myosin VI puncta in the peripheral domain colocalize with each other [Fig. 4(L); for quantification see Fig. 5(A)]. In the central domain, the degree of myosin V and VI puncta colocalization is difficult to estimate, because the puncta are densely
packed in this region that is thicker than the peripheral domain [Fig. 4(L)]. When myosin V and VI were detected with the same secondary antibody on different cultures, the stainings had similar intensities, myosin V appearing somewhat more abundant (data not shown). The punctate distribution of myosin V and myosin VI suggests that these myosins might be associated with organelle structures in the growth cone, although this may not be true for all puncta, as found in other myosin localization studies (Lewis and Bridgman, 1996; Evans et al., 1997). However, in some growth cones (Fig. 3) we observe that a fraction of these myosins (especially of myosin VI) can exhibit a more diffuse staining pattern, suggesting a cytosolic protein fraction.

Further analysis of the myosin V and myosin VI distribution in growth cones was carried out with respect to the major cytoskeletal structures, F-actin and microtubules (Fig. 3). Both myosins are found in the same subcellular region as microtubules in the central domain [Fig. 3(C,D) and (G,H)]. In the peripheral domain, myosin V is present in F-actin-containing lamellipodia and filopodia [Fig. 3(A,B)], whereas myosin VI shows very little overlap with F-actin structures [Fig. 3(E,F)].

**Subcellular Association of Myosin V and Myosin VI in Growth Cones**

We further investigated the subcellular association state of myosin V and myosin VI in growth cones by three different types of experiments: (1) live cell extraction, (2) nocodazole treatment of cultured DRG neurons, and (3) Western blot analysis of growth cone particle preparations.

Live cell extraction experiments should help to
determine whether there is a significant fraction of readily extractable, presumably cytosolic, myosin V or myosin VI [Fig. 4(A–K)]. DRG neurons were treated for 30 s with 0.02% saponin followed by fixation in the presence of 1% Triton X-100 (see Materials and Methods). Figure 4(A–C) shows the structural changes during such an experimental treatment visualized by high-resolution DIC video microscopy. Saponin, which disrupts the plasma membrane, causes a "fuzzy" appearance of the growth cone cell surface when observed in DIC [Fig. 4(B)]. This "fuzzy" look of the growth cone disappeared after further removal of membranes by Triton X-100 during the fixation process [Fig. 4(C)].

Purlyy cytosolic proteins should not be detected in significant amounts by immunocytochemistry using an extraction procedure as described above. In contrast, nonextractable protein complexes such as microtubules and F-actin should not be affected by this treatment. Indeed, microtubule and F-actin staining [Fig. 4(D–F)] were indistinguishable from control stainings during which cells were extracted after fixation [Fig. 3(A,C,E,G)]. Interestingly, the staining pattern of myosin V and myosin VI after live cell extraction [Fig. 4(E,G,H,I,K)] was similar to the control condition [Fig. 2(C,D), Fig. 4(L)], with a higher concentration of puncta in the central domain than in the peripheral regions of the growth cone. However, quantification of myosin V and VI puncta in the peripheral domain, where individual puncta can be better resolved, revealed a 48% reduction of single-labeled myosin V puncta (33 ± 6 versus control 64 ± 9 puncta; t-test, p < .01) and a 71% reduction of single-labeled myosin VI puncta (19 ± 5 versus control 65 ± 17 puncta; t-test, p < .01) when compared with control conditions [Fig. 5(B)]. Double immuno-
staining for myosin V and myosin VI after live cell extraction revealed a partial colocalization of these myosins in the growth cone [Fig. 4(H,K,L)] as shown under normal staining conditions [Fig. 4(L)]. Interestingly, double-labeled puncta in the peripheral domain were more resistant to saponin extraction than single-labeled puncta and were reduced only by 30% ± 19 ± 2 versus control (13 ± 2 puncta; t-test,  p > .01; Fig. 5[B]). These differences in extractability between single- and double-labeled puncta, especially in the case of myosin VI, result in a relative increase of myosin VI in double-labeled puncta after live cell extraction [Fig. 5(A)]; 34 ± 5% versus control 18 ± 3% of total myosin VI in double-labeled puncta; t-test,  p < .01]. Taken together these experiments suggest that a significant portion of both myosin V and myosin VI in the central domain appear to be in relatively tight association with either cytoskeletal or organelle structures. In the peripheral domain, myosin V has a higher association than myosin VI to such structures.

We further investigated and quantified the proportion of soluble myosin V and VI in total embryonic (E10) chick brains as well as in growth cone particles isolated from these brains (Fig. 6). Total brains were homogenized and fractionated by differential centrifugation using the same buffer as in the live cell extraction experiment [Fig. 6(A)]. The amounts of myosin V and VI in each fraction were then analyzed by quantitative Western blotting. The fractionation profile of these two myosins is different after the ultra-high-speed centrifugation step. In agreement with previous findings using 1-day-old brain tissue and different homogenization conditions (Cheney et al., 1993; Evans et al., 1998) the majority of myosin V is found in the 120,000 × g sedimentation fraction containing small organelles [Fig. 6(A); USP]. In contrast, only 37% of the myosin VI was sedimentable at 120,000 × g, whereas 63% remain cytosolic [Fig. 6(A); USS]. These results suggest that in total brain a significant amount of myosin VI is not associated with sedimentable structures such as cytoskeletal filaments and/or organelles.

To better compare the biochemical data with the immunofluorescence growth cone data after live cell extraction, we isolated growth cone particles from embryonic (E10) chick brains according to Pfenninger et al. (1983). Both myosin V and VI can be detected in this growth cone particle preparation; however, they are not enriched when compared with the homogenate [Fig. 6(B)]. In contrast, we found enrichment of GAP-43, which we used as a marker protein for our growth cone particle preparation. As a second control for our growth cone particle isolation, we visualized the particles by high-resolution DIC optics (data not shown). The majority of the particles had a diameter of 0.5–1.5 μm, which is in agreement with their original characterization (Pfenninger et al., 1983). Myosin VI can be saponin-extracted from the growth cone particles to 42% [Fig. 6(B)], whereas extracted myosin V can only be detected after long blot exposures (1–3 min; data not shown). To summarize, myosin VI is more readily extractable than myosin V from growth cone particles as well as from total brain, suggesting a difference in their association state with subcellular structures.

Both unconventional myosins are highly concentrated in the central domain of the growth cones where microtubules are the major cytoskeletal structures (Fig. 3). To investigate a potential association of myosin V and myosin VI with microtubules, we treated the neuronal cultures with the microtubule-
Figure 2  Expression of myosin V and myosin VI in cultured DRG neurons. DRG neurons cultured on laminin substrata were fixed after 20 h and immunostained for myosin V (A, C) and myosin VI (B, D). Both myosin V (A) and myosin VI (B) are localized in the neuronal cell body, neurites, and growth cones. A more detailed view of myosin V and myosin VI distributions in the growth cone are shown in (C) and (D). Both myosin V and myosin VI exhibit a punctate distribution with its highest concentration in the central domain of the growth cone. Bars: (A, B) 25 μm; (C, D) 5 μm.

determine whether there is a significant fraction of readily extractable, presumably cytosolic, myosin V or myosin VI [Fig. 4(A–K)]. DRG neurons were treated for 30 s with 0.02% saponin followed by fixation in the presence of 1% Triton X-100 (see Materials and Methods). Figure 4(A–C) shows the structural changes during such an experimental treatment visualized by high-resolution DIC video microscopy. Saponin, which disrupts the plasma membrane, causes a “fuzzy” appearance of the growth cone cell surface when observed in DIC [Fig. 4(B)]. This “fuzzy” look of the growth cone disappeared after further removal of membranes by Triton X-100 during the fixation process [Fig. 4(C)].

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Figure 4  Myosin V and myosin VI puncta in the central domain are resistant to live cell extraction. (A–C) Video-enhanced DIC images showing a DRG growth cone on laminin substrate during the process of a live cell extraction experiment. (A) Live in culture medium. (B) After a 30-s treatment with 0.02% saponin in PHEM buffer. (C) After fixation in the presence of 1% Triton X-100. (D–K) Immunolocalization of myosin V (E and G, rabbit anti-myosin V head antibody; H, rat anti-myosin V head antibody) together with tubulin (D), F-actin (F), and myosin VI (L, rabbit anti-myosin VI tail antibody) reveals that the punctate staining pattern of both myosins remains in the central domain after live cell extraction. (K) Pseudocolor overlay of the myosin V (green) and myosin VI (red) double staining showed in (H) and (I), respectively. Some of the puncta are colocalized (arrow). (L) Pseudocolor overlay of myosin V (green) and myosin VI (red) double staining using normal fixation protocol (no live cell extraction) reveals only partial colocalization of myosin V and myosin VI puncta in the peripheral domain (arrow). Bar: 5 μm.

depolymerizing drug nocodazole (1 μM) for 1 h (Fig. 7). Such treatment resulted in disruption of microtubule structures in the central and peripheral domain of the growth cone [Fig. 7(C,E)] compared to control growth cones [Fig. 7(A)], whereas higher doses of nocodazole had more severe effects on growth cone morphology, making myosin V and VI localization difficult. The effect of the nocodazole treatment also is demonstrated by the diffuse tubulin immunofluorescence staining pattern in both lamellipodia and filopodia [Fig. 7(C,E)], suggesting higher levels of free tubulin relative to control growth cones [Fig. 7(A)]. However, disruption of microtubules in the growth cones did not appear to affect myosin V or myosin VI localization significantly [Fig. 7(D,F)]. Interestingly, nocodazole-treated growth cones had longer filopodia than control growth cones containing both myosin V and VI puncta. To summarize, the results of the nocodazole experiments suggest that distribution of these myosins within the growth cone does not require direct interaction with microtubules.
Figure 5 Quantification of myosin V and myosin VI puncta in peripheral growth cone domains. Single- and double-labeled myosin V and VI puncta were counted in the peripheral domain of growth cones under control (n = 60) and live cell extraction condition (n = 5; see Methods; average values ± S.E.M. are given). (A) Relative puncta distribution of single- and double-labeled puncta for each myosin. Note: Increase in the relative fraction of myosin VI label in double-labeled puncta after live cell extraction. (B) Reduction of the number of myosin V and VI puncta in the peripheral growth cone domain after live cell extraction.

DISCUSSION

Localization of Myosin V and Myosin VI in Growth Cones

In this study, we found that both myosin V and myosin VI are highly expressed in the chicken brain and DRG during embryonic development. Our myosin V data are in agreement with previous studies on myosin V expression (Mercer et al., 1991; Espinol et al., 1992; Espinol and Mercer, 1992). In contrast, the expression pattern of myosin VI has not been well characterized to date. With respect to the nervous system, myosin VI has been localized in the sensory hair cells of the inner ear (Avraham et al., 1995; Hasson et al., 1997), and very recently the Drosophila myosin VI homologue, 95F, has been detected in axons of the central nervous system in fly embryos (Lantin and Miller, 1998).

In the growth cone, both myosin V and myosin VI exhibit a punctate staining pattern that is concentrated in the central domain. Here, both myosins are present in the same subcellular region as microtubules. The punctate staining patterns suggest that both myosins

Figure 6 Myosin V and myosin VI are differentially associated with growth cone particles. (A) Myosin V and VI from embryonic brain exhibit different fractionation patterns. Whole brains from chick embryos (E10) were homogenized and fractionated by differential sedimentation (see Methods). Stoichiometric samples from each fractionation step were separated on 5-20% SDS-PAGE and included the whole brain homogenate (HOM), the low-speed supernatant (LSP), high-speed supernatant (HSP), and ultra-speed supernatant (USS). Western blots were probed with rabbit anti-myosin V (top row) and rabbit anti-myosin VI tail antibody (bottom row), respectively. A major difference between myosin V and myosin VI was detected in ultra-speed centrifugation step: myosin V is found predominantly in the pellet fraction (USP), whereas a higher fraction of myosin VI is found in the supernatant (USS). (B) Myosin V and VI are differentially extracted from growth cone particle preparations. Growth cone particles were isolated from whole embryonic chick brains (E10; see Methods). Equal protein amounts from each fractionation step were separated on 5-20% SDS-PAGE and included the following samples: homogenate (HOM), low-speed supernatant (LSP) and pellet (LSP), growth cone particles (GCP), supernatant (GCEP), and pellet (GCEP) after supernat extraction of growth cone particles. Western blots were probed with rabbit anti-myosin V tail, rabbit anti-myosin VI tail and mouse anti-GAP-43 antibody, respectively. Myosin V and VI are present, but not enriched in growth cone particles.
Figure 7  The punctate distributions of myosin V and VI within the growth cone are not altered by the disruption of microtubules. DRG growth cones cultured under control conditions (A, B) and after treatment with 1 µM nocodazole for 1 h (C–F) were double stained for tubulin (A, C, E) and myosin V (B, D) and myosin VI (F). Nocodazole treatment caused a disruption of microtubule structures within the growth cone (C, E), but did not change the punctate myosin V (D) and myosin VI (F) distribution significantly. Bar: 5 µm.

may be associated with vesicular structures; however, this may not be true for all puncta, as recently demonstrated in other myosin localization studies (Lewis and Bridgman, 1996; Evans et al., 1997). Our findings on myosin V distribution in growth cones of cultured chick DRG neurons is in complete agreement with the results of a recent study on myosin V localization in growth cones of rodent superior cervical ganglia neurons (Evans et al., 1997). Evans et al. detected myosin V immunogold-labeling on organelles that are associated with microtubules. However, a direct microtubule association was not found, which is in agreement with the result of the nocodazole experiment in our study as well as that done by Evans et al. (1997).

This study is the first to show localization of a class VI myosin in growth cones. The presence of myosin VI in the microtubule-rich central domain is important to note, since it was recently found that Drosophila
myosin VI coimmunoprecipitates with a microtubule-binding protein D-CLIP-190 (Lantz and Miller, 1998). Although myosin VI exhibits a similar punctate distribution in the growth cone as myosin V, we found only partial colocalization in the peripheral domain (18% of all myosin VI puncta in double-labeled puncta), suggesting that a significant portion of these myosins may be associated with distinct vesicular or cytoskeletal structures. In addition, the live cell extraction experiments revealed that these myosins differ in their association state at least in the peripheral domain, where individual puncta can be analyzed. The higher extractability of myosin VI puncta from the peripheral domain (Fig. 5) is in agreement with the results of our biochemical fractionation of total brain tissue as well as of our growth cone particle preparation (Fig. 6). These results indicate that a larger fraction of myosin VI than of myosin V in total brain tissue and growth cones is cytosolic and/or only weakly associated with cytoskeletal/organelle structures. Differences in the levels of extractability, when comparing the three methods for the same myosin, could be explained both by the different starting material used for quantification (peripheral domain of cultured growth cones, total brain, growth cone particles), and by the different technical approaches. To summarize, all of our results suggest that a major fraction of both myosin V and VI have distinct subcellular distributions in growth cones.

With which organelles are these myosin motors associated? Two sets of biochemical studies indicate that myosin V is in part associated with membranes containing synaptic vesicle proteins (Prekeris and Terrian, 1997; Evans et al., 1998). Myosin V is present in preparations of synaptic vesicles and can be biochemically crosslinked to the synaptic vesicle proteins synaptobrevin I and synaptophysin (Prekeris and Terrian, 1997). However, immuno-electron microscopic analysis revealed that the majority of vesicles containing both myosin V and the synaptic vesicle protein, SV2, are much larger (30-250 nm) than mature synaptic vesicles (50 nm), which mostly lack myosin V (Evans et al., 1998). Thus, myosin V may be associated with organelles involved in synaptic vesicle maturation or recycling (Evans et al., 1998). Consistent with the fractionation results of the present study, myosin VI is not enriched in the myosin V/synaptic vesicle–enriched membrane fractions described by Evans et al. (1998; L. Evans and M. Mooseker, unpublished observations).

**Functions of Myosin V and Myosin VI in Growth Cones**

Myosins of four different classes have been identified in growth cones so far: I, II, V, and VI. Considering the growing myosin superfamily, it is quite possible that myosins of other classes might be localized in this highly specialized neuronal structure. Why are there so many different myosin motors in growth cones and in cells in general? It has become evident that myosins are involved in a variety of cellular functions, such as cell movement, membrane traffic, and signal transduction in different systems (for reviews, see Titus, 1997; Mermall et al., 1998). There is biochemical, genetic, immunocytological, and physiological evidence, suggesting that myosin II and probably myosin I are involved in growth cone motility, whereas myosin V may play a role in organelle transport (reviewed in Hasson and Mooseker, 1997).

Is myosin V just a passenger or actively involved in organelle movement in growth cones? Recent functional studies on brain vesicle–associated myosin V revealed that these organelles are capable of actin-based transport that can be blocked by antibodies against the myosin V motor domain (Evans et al., 1998). A potential role for myosin V in membrane delivery could explain the effects on filopodial dynamics when myosin V protein is locally inactivated in growth cones (Wang et al., 1996). The idea of a myosin acting as an organelle motor in neurons is particularly intriguing since there have been several reports on actin-based organelle transport in the past (Kuznetsov et al., 1992; Bearer et al., 1993; Langford et al., 1994; Evans and Bridgman, 1995; Morris and Hollenbeck, 1995; Evans et al., 1998; Tabb et al., 1998). Thus, there is an interesting possibility that organelles are transported along microtubule tracks by action of kinesin and dynein motors inside the axon, but when they arrive in the growth cone peripheral domain, they switch the transport machinery to the actin/myosin system to achieve their final destination. The recent findings on the direct interaction between myosin Va and kinesin provide further evidence for the functional cooperation of these two motor systems (Huang et al., 1999).

The punctate staining pattern of myosin VI is similar to the one of myosin V, suggesting that myosin VI may also be associated with organelles in the growth cone. There is evidence that myosin VI might be an organelle motor in *Drosophila* embryos and might be involved in cytoplasmic transport during oocyte maturation (Mermall et al., 1994; Mermall and Miller, 1995; Bohmann, 1997; Lantz and Miller, 1998). However, the function of myosin VI as an organelle
motor needs to be further analyzed, perhaps with an in vitro motility assay similar to the one used for myosin V (Evans et al., 1998). Obtaining more information on myosin VI function will help our understanding of why there are so many different myosins in neurons.

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